

Prognostic significance and diagnostic value of PTEN and p53 expression in endometrial carcinoma. A retrospective clinicopathological and immunohistochemical study

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Summary

Purpose: To investigate the PTEN and p53 gene expression in endometrioid and serous papillary endometrial carcinomas and clarify their prognostic significance by studying the PTEN and p53 expression in relation to tumor stage and grade.

Methods: Archival pathological sections of 61 cases with endometrial cancer examined in a 5-year-period (January 2006-December 2010) were retrieved and re-examined. Immunohistochemical investigation was performed by the Ventana system. Anti-PTEN and anti-p53 monoclonal antibodies were used. Disease staging was made according to the FIGO staging system.

Results: Forty-nine (80.32%) cases were endometrioid adenocarcinomas. Patient age ranged from 39-75 years (mean 62.5). Grade 1 tumors: 19/22 (86.3%) cases had stage Ib, 2/22 (9.09%) stage Ic and 1/22 (4.54%) stage IIIc. Eighteen of 22 (81.8%) cases were PTEN positive and 4/22 (18.2%) p53 positive. Grade 2 tumors: 17/23 (73.91%) cases had stage Ib, 4/23 (17.39%) stage Ic and 2/23 (8.69%) stage IIIc. Seventeen of 23 (73.91%) cases were PTEN positive and 4/23 (17.3%) p53 positive. Grade 3 tumors: 2/4 (50%) cases had stage Ic and 2/4 (50%) stage IIIc. No case was PTEN positive and 2/4 (50%) were p53 positive. Twelve (19.35%) cases were serous papillary carcinomas. Patient age ranged from 63-79 years (mean 76). Five (41.66%) cases had stage Ic and 5 (41.66%) stage IIIc, with nodal metastases and peritoneal involvement. Two (16.66%) cases developed on endometrial polyps with minimal myometrial involvement (stage Ib) and in both cases elements of endometrioid adenocarcinoma were observed as well. Immunohistochemical study showed that 11 (91.66%) cases were p53 positive and 2 (16.66%) PTEN positive.

Conclusion: PTEN and p53 immunorexpression helps both in accurate diagnosis and proper therapeutic approach of the various endometrial carcinomas. PTEN and p53 are also prognostic markers for these kind of tumors.

Key words: endometrial adenocarcinoma, endometrioid adenocarcinoma, serous papillary adenocarcinoma, p53, PTEN

Introduction

Endometrial carcinoma is one of the most common gynecological malignancies in the developed countries and predisposing factors like hypertension, diabetes mellitus, late menopause, estrogen replacement treatment, and obesity have been associated with this disease [1]. Endometrial cancer occurs in premenopausal women (25%) and postmenopausal women (75%) and the most common age group is between 50-59 years. In about 5% of all cases, endometrial adenocarcinoma is considered to be related with hereditary predisposition, particularly when tumors present in individuals younger than 55 years [1].

As the development of the disease in most cases is strongly associated to estrogen action, a dual model of carcinogenesis has been established: a) Type I carcinoma, with its most common form being the endometrioid adenocarcinoma, which is a low grade, estrogen-dependent neoplasm, usually arising in the early postmenopausal age group; and b) Type 2 carcinoma, with its most common form being the uterine serous papillary carcinoma, which is not estrogen-dependent and arises in elderly postmenopausal women [1-3]. About 70-80% of type I endometrial adenocarcinoma cases occur on a background of endometrial hyperplasia, and the endometrial changes of endometrial intraepithelial neoplasia are considered as precursor lesions. Serous papillary tumors arise on atrophic endometrium [4-6].

The prognosis of type I endometrial cancer differs, depending on the type, grade and depth of myometrial invasion, and stage. Type 2 endometrial cancers are aggressive tumors and generally present a poor prognosis [1,3]. In the last decades, the progress made in analyzing the human genome identified additional factors, particularly in molecular and cellular level concerning the pathogenesis of endometrial carcinoma. Specifically, several DNA mutations concerning proteins involved in mechanisms of cell signal transduction and communication have been investigated. Among them, the most frequently observed gene mutation is located on chromosome 10 and is related with the PTEN gene (phosphatase and tensin homolog) [7,8].

The PTEN gene belongs to the family of tumor

suppressor genes, which are involved in cell cycle regulation and cell proliferation and are also involved in apoptotic cell processes. There are two main pathways through which apoptosis occurs: a) the extrinsic or cytoplasmic pathway which is activated by the death receptor FAS (CD 95), which belongs to the TNF receptors; and b) the intrinsic pathway, which is regulated by Bcl2 protein, resulting in the release of cytochrome-C from the mitochondria. Then, both pathways activate the caspase intracellular proteins, which are proteases leading to cell apoptosis. Mutations of PTEN gene could cause decreased production of proteins involved in the apoptotic pathways, like the serine-threonin kinase [9,10]. It has been reported that the majority of the above described genetic changes that occur in the endometrioid type of endometrial carcinoma reflects very early stages of carcinogenesis, but it is not yet fully clarified which of these alterations are really the first signs of malignant transformation [7,8].

p53 protein is involved in gene transcription, DNA synthesis and repair, genomic plasticity, and apoptosis. p53 is observed in serous papillary endometrial carcinomas, but is also observed in 10-15% of early and 40-50% of advanced endometrioid endometrial carcinomas [11]. Its identification is correlated with poor prognosis and the mutations of p53 gene and overexpression of Her2/Neu are expressed with early changes in tumors of low differentiation [11,12].

The aim of this study was to investigate the PTEN and p53 gene immunopexpression in cases of endometrioid and serous papillary endometrial carcinoma, and clarify their prognostic significance by analysing the correlation between PTEN and p53 expression with tumor grade and disease stage.

Methods

Archival histological sections of 61 cases of endometrial cancer examined in a 5-year period (January 2006-December 2010) in the Pathology Laboratory of "Aretaieion" University Hospital were retrieved and re-examined. Clinical and additional data were obtained from the files of the 2nd and 3rd Department of Obstetrics and Gynecology of University of Athens in Aretaieion and Attikon Hospital of Athens.

Permission for this study was obtained from the Research and Ethics Committee of Aretaieion Hospital. All patients were investigated by diagnostic dilatation and curettage before definite surgery, due to abnormal vaginal bleeding and/or transvaginal ultrasound (U/S) signs of suspicious endometrial malignancy. All studied patients underwent total abdominal hysterectomy with bilateral adnexectomy and in 16 cases pelvic lymph node resection was performed because of the extensive infiltration of the myometrium diagnosed by frozen section studies during surgery.

FIGO staging system was used to characterize disease stage.

All specimens were fixed in 10% neutral formalin solution and processed routinely. Pathological examination included clarification of cancer type, grade of tumor differentiation, myometrial and vessel invasion and the presence of lymph node metastases. Immunohistochemistry was performed by the Automatic Ventana System (Tucson, Arizona, USA). Anti-PTEN monoclonal antibody (Biocare, Concorde, CA, USA, clone 6H2.1) and anti-p53 monoclonal antibody (clone DO7, DAKO Corporation, Denmark) were used according to the manufacturer's instructions. Positive immuno reaction was graded semi quantitatively: cases with positive p53 immunoreaction in >10% of the nuclei of neoplastic cell population, and positive PTEN immunoreactions in >80% of neoplastic glandular cells, were considered positive (+), while all other cases were considered negative (-).

Statistics

Statistical analysis was performed with SPSS, version 20 (IBM, USA). The level of significance was set < 5%. Pearson's χ^2 test was used to compare the expression of PTEN and p53 with grade and stage for endometrioid and serous papillary adenocarcinomas.

Results

Clinicopathological and immunohistochemical study of endometrioid adenocarcinomas

Of 61 cases examined 49 (80.32%) were endometrioid endometrial adenocarcinomas. Patient age ranged from 39-75 years (mean 62.5). Stage and grade in relation to PTEN and p53 expression are shown in Table 1. PTEN expression was positive in 71.4% of the cases (35 patients), while p53 expression was positive in 18.4% of the cases (9 patients). Grade was related with PTEN expression since positive PTEN expression correlated mainly with grade I (81.8%) and 2 (73.9%) and negative expression with grade 3 (100%; $p=0.004$). In contrast, grade did not correlate with p53 expression ($p=0.936$). Both PTEN and p53 expressions were correlated with stage ($p<0.0001$ and $p=0.001$, respectively). Positive PTEN expression was correlated with stage IB (88.9%), while negative expression with stages IC and IIC (75% and 80%, respectively). Positive expression of p53 correlated with stage IIIC (80%) while negative expression with stages IB (86.1%) and IC (100%). In 45 out of 61 (73.8%) cases the non neoplastic endometrium displayed hyperplastic changes: moderate (35 cases) and severe (7

Table 1. PTEN and p53 immunoreaction in 49 cases of endometrioid adenocarcinomas according to tumor grade and stage

		PTEN		<i>p</i> -value	p53		<i>p</i> -value
		Positive N (%)	Negative N (%)		Positive N (%)	Negative N (%)	
Grade	1	18 (81.8)	4 (18.2)	0.004	4 (18.2)	18 (81.8)	0.936
	2	17 (73.9)	6 (26.1)		4 (17.4)	19 (82.6)	
	3	0 (0.0)	4 (100)		1 (25.0)	3 (75.0)	
Stage	Ib	32 (88.9)	4 (11.1)	<0.0001	5 (13.9)	31 (86.1)	0.001
	Ic	2 (25.0)	6 (75.0)		0 (0.0)	8 (100)	
	IIIC	1 (20.0)	4 (80.0)		4 (80.0)	1 (20.0)	
Total		35 (71.4)	14 (28.6)		9 (18.4)	40 (81.6)	

cases), while in 3 cases an irregularly proliferative endometrium was observed. Figure 1 shows a case of endometrioid carcinoma with PTEN positive expression.

Clinicopathological and immunohistochemical study of serous papillary adenocarcinomas

Twelve out of 61 cases (19.35%) had endometrial serous papillary carcinomas. The patient age ranged from 63-79 years (mean 76). Two of 12 cases (16.66%) developed on endometrial polyps with minimal myo-

metrial involvement (stage IB) and in both cases elements of endometrioid adenocarcinoma were observed as well. All cases developed in atrophic endometria, with the exception of the above two mentioned cases where a mixed neoplastic pattern was observed on endometrial polyps.

Disease stage in relation to PTEN and p53 expression is shown in Table 2.

This study showed that 11/12 cases (91.66%) were p53 positive (Figure 2) and 2/12 cases (16.66%) were

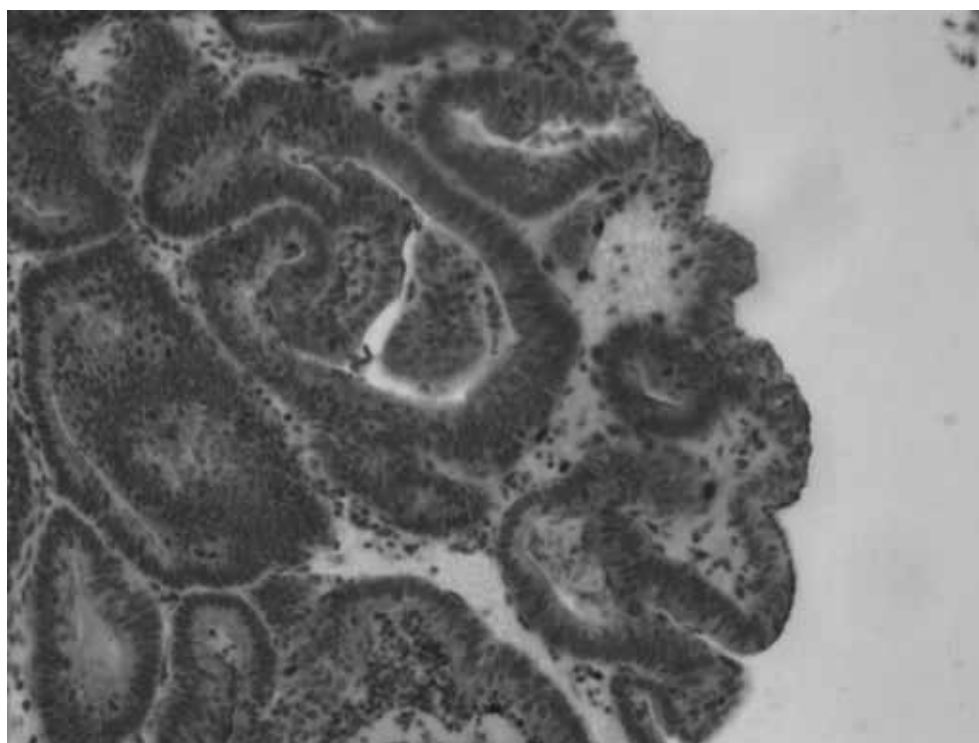


Figure 1. Histological section of an endometrioid endometrial adenocarcinoma presenting extensive PTEN+immunoreaction (immunostain, x250).

Table 2. PTEN and p53 immunoreaction in 12 cases of serous papillary endometrial adenocarcinomas according to stage

		PTEN		<i>p-value</i>	P53		<i>p-value</i>
		Positive	Negative		Positive	Negative	
		<i>N (%)</i>	<i>N (%)</i>		<i>N (%)</i>	<i>N (%)</i>	
Stage	Ib	1 (50.0)	1 (50.0)	0.267	2 (100)	0 (0.0)	0.466
	Ic	1 (20.0)	4 (80.0)		5 (100)	0 (0.0)	
	IIIc	0 (0.0)	5 (100)		4 (80.0)	1 (20.0)	
	Total	2 (16.7)	10 (83.3)		11 (91.7)	1 (8.3)	

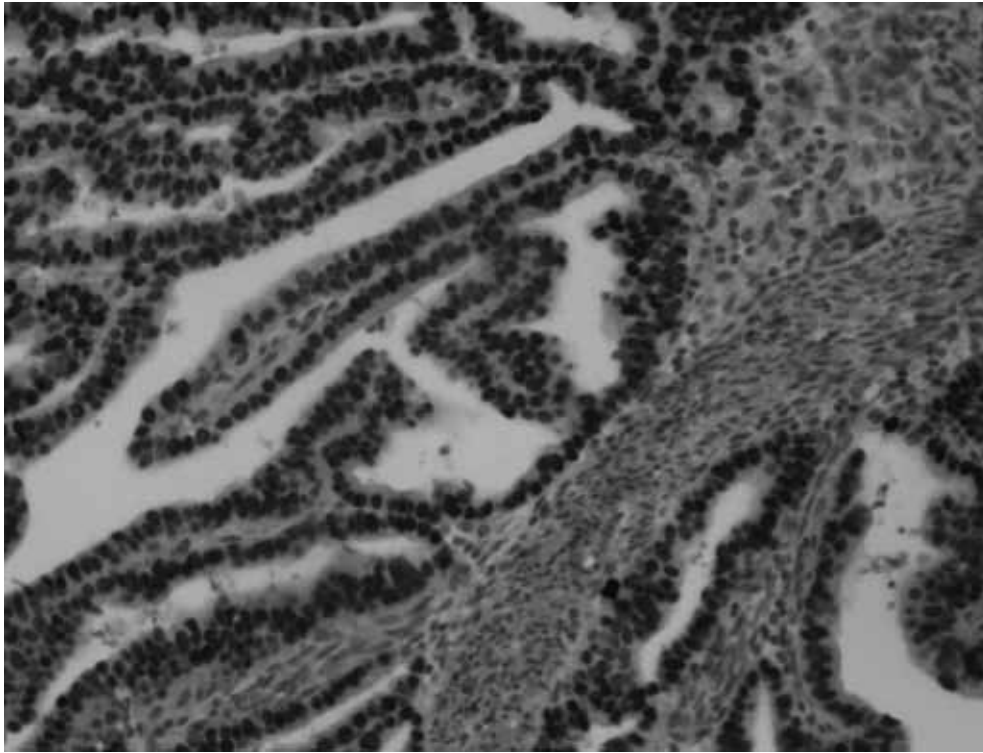


Figure 2. Histological section of serous papillary endometrial adenocarcinoma with extensive p53 nuclear immunoreaction (immunostain, x250).

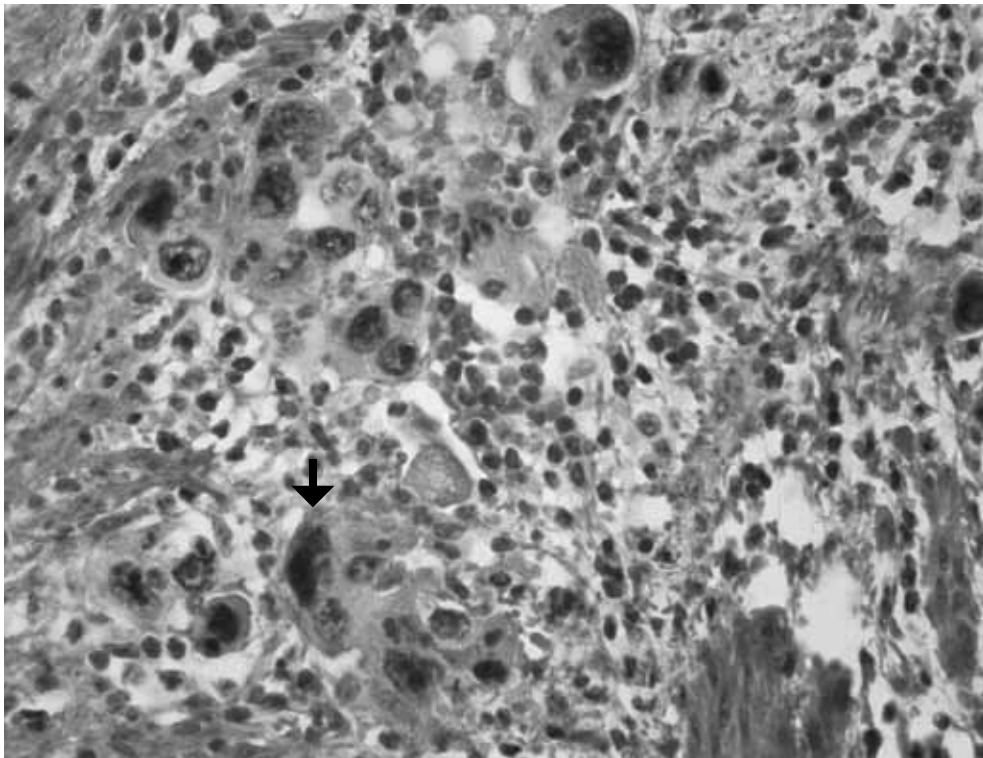


Figure 3. Histological section of myometrium showing small clusters of p53 positive neoplastic cells (arrow) among inflammatory cells (immunostain, x400).

PTEN positive. However, stage was not correlated with either PTEN ($p=0.267$) or p53 ($p=0.466$). Isolated p53 positive neoplastic cells were observed infiltrating the myometrium and thin-walled vascular spaces, not recognized in sections stained by hematoxylin-eosin (Figure 3) and one case was up-staged due to this finding.

Discussion

The genomic profile of gynecological malignancies provides a new field of investigation in order to achieve earlier diagnosis, and better prognosis of endometrial cancer. Previous studies have shown that p53 and/or PTEN signaling pathways might play an important role in the pathogenesis of endometrial carcinoma [4-8].

Different tumor suppressor genes like PTEN, p53 and K-ras have been investigated in support of the hypothesis that type I and type II endometrial adenocarcinoma presents different molecular carcinogenetic pathways [13,14]. PTEN represents the most important genomic predisposing factor in the development of endometrioid endometrial adenocarcinoma and PTEN inactivation is an early event in carcinogenesis as well as the most frequent abnormality in type I carcinoma [10]. Garg et al. [7] showed that evaluation of PTEN loss by immunohistochemistry is highly reproducible with the application of standard immunohistochemical techniques and simple scoring criteria [14]. PTEN gene is located at chromosome 10q23 encoding a protein and lipid phosphatase which acts as tumor suppressor. PTEN inactivation is induced by different mutations. PTEN is implicated in different pathways of carcinogenesis including cell cycle arrest at the G1/S checkpoint, AKT-dependent mechanisms mediated by PTEN, downregulation of anti-apoptotic mechanisms through Bcl-2 [11-13], and opposition to PI3K [14,15]. Moreover, PTEN is implicated in the inhibition of focal adhesion formation, cell spread, migration, and inhibition of growth-factor-stimulated MAPK signaling [16,17]. PTEN mutations are reported in 25–83% of tumors, more frequently in endometrioid carcinomas, as an early event in the pathogenesis pathway. According to Kim et al. [15] PTEN and K-ras double-mutant mice (Ptend/dK-rasG12D) exhibit an acceleration of endometrial

carcinoma development compared to carcinomas formed from a single PTEN or K-ras gene mutation. These results suggest a synergistic effect of PTEN and K-ras signaling pathways during endometrial carcinogenesis. p53 mutation is involved in 90% of type II carcinoma [13]. p53 gene is located on chromosome 17 and is important in preventing the proliferation of cells with damaged DNA. p53 mutations or TP53 overexpression is twice as frequent in type II tumors, is observed in 17% of grade III endometrioid carcinomas [18-20] and is correlated with non-endometrioid histological type, high-grade tumors, and absence of the progesterone receptor [21,22]. According to Zheng et al. [11] p53 mutations are an early event in the development of serous papillary carcinoma and early p53 nuclear accumulation may help identify precursor lesions of serous papillary carcinoma [22]. Our data agree with the current evidence concerning the relationship between grade/stage and PTEN overexpression in the endometrioid endometrial adenocarcinoma. In our study p53 overexpression was independent of the stage of the disease and observed in two early cancers which developed on endometrial polyps. This distinct immunoprofile of endometrioid and of serous papillary endometrial carcinoma is a diagnostic and prognostic marker. Of interest is our finding of two cases of endometrial polypoid adenocarcinoma with mixed pattern, both endometrioid (moderately differentiated) and serous papillary with distinct immunopathological features.

In conclusion, the immunoeexpression of PTEN and p53 is a helpful aid both in the accurate diagnosis of the various endometrial carcinomas and the proper therapeutic approach, and are also prognostic markers, since the morphological type and the immunoprofile of an endometrial neoplasm present definite prognostic information.

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Prognostic significance of matrix metalloproteinase-2 in gynecological cancer: a systemic review of the literature and meta-analysis

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Summary

Purpose: Matrix metalloproteinases (MMPs) are considered as mediators of metastases which may be associated with gynecological cancer survival. However, such relationship remains inconclusive. We carried out the present metaanalysis to evaluate the prognostic value of MMP-2 and MMP-9 in gynecological cancers.

Methods: We searched 2 medical databases (Medline and Embase) and located 13 studies with 1841 patients that evaluated the relationship between MMP-2 and MMP-9 and 5-year survival. Risk ratio (RR) with 95% confidence intervals (95% CI) synthesized by random effect model were used to assess the strength of the association. Publication bias was evaluated by Begg-Mazumdar test and Egger's regression test.

Results: Mortality was 1.53-fold higher in patients whose tumor cells were positive for MMP-2 (RR 1.53; 95% CI 1.03-2.27; $p=0.03$). Funnel plot was symmetrical ($p=0.721$ for Begg-Mazumdar test, and $p=0.718$ for Egger's regression test). Between-study heterogeneity was significant ($p<0.001$). Mortality was 1.26-fold higher in MMP-9 positive than negative patients, but without statistical difference (RR 1.26; 95% CI 0.94-1.68; $p=0.12$). Funnel plot was asymmetrical ($p=0.024$ for Begg-Mazumdar test).

Conclusion: MMP-2 positivity in tumor cells is associated with worse survival in patients with gynecological cancers. Standardization of MMP positivity is needed.

Key words: matrix metalloproteinase, meta-analysis, ovarian cancer, overall survival

Introduction

Gynecological cancers are the leading cause of death in women. Despite development in diagnosis and treatment of gynecological cancers, the survival rate remains largely unchanged, especially in ovarian cancer. There are several common prognosis related factors such as FIGO stage, histological grade, CA-125, CIP2A, VEGF-A, Ets transcription factor, survivin, but actually few of these factors are routinely used in clinical practice [1-4], raising the need to find better markers that can identify patients with poor prognosis.

Extracellular matrix (ECM) degradation plays a predominant role in extracellular microenvironment homeostasis. Irregular proteolysis of ECM leads invariably to unregulated tumor growth, tissue remodeling, inflammation, tissue invasion, and metastasis. MMPs are a family of zinc-dependent proteolytic enzymes that are constantly correlated with other cellular and extracellular proteins, assumed to play a key role in a variety of physiological and pathological conditions [5,6]. Thorough and persistent investigations have clearly recognized that MMPs not only control the ECM turnover and cancer cell migration, but also regulate signaling pathways of cell growth, morphogenesis, angiogenesis, tissue repair and metastasis [7,8]. Gelatinolytic activity of MMP-2, a 72 kDa type IV collagenase, has been associated with malignant phenotype of different solid neoplasms. Switch from the initial form of pro-MMP-2 (72 kDa) to enzymatic MMP-2 (62 kDa), which is mediated by intracellular furin-like proteinases, is essential to its proteolytic activity. Owing to its unique ability to degrade type IV collagen, the major component of ECM and basement membrane, tumor cells can easily penetrate the ECM and spread locally and/or distantly (metastasis) [9]. The level of MMP-2 was found to correlate with the metastatic potential of numerous cell types [10]. MMP-9 is a 92 kDa type IV collagenase, also called gelatinase B, which can degrade collagen type IV, like MMP-2 [11].

Recently, several studies reported association of MMPs with prognosis of gynecological cancers (Table 1). Therefore, it is necessary to explore whether MMP-2 or MMP-9 expressions are prognostic factors in gynecological cancers.

Due to differences in study populations and designs, the results of some studies are inconclusive. Our aim was to prove the hypothesis that MMP-2 or MMP-9 are connected with 5-year overall survival in gynecological cancers. Thus, we conducted a meta-analysis of all available studies relating MMP-2 or MMP-9 with the prognostic outcome in patients with such malignancies.

Methods

Publication search

Initially, we performed an online search in PubMed and Embase to identify all related studies (between January 1990 and May 31, 2011), regardless of the MMP subtype and publication language in patients with gynecological cancers. The search was carried out using the following keywords: "MMP" or "metalloproteinase", "gynecological cancer", "ovarian cancer", "cervical cancer", "endometrial cancer", with no special limits except time. Then we used Endnote (version 4.1) to screen the literature including all of the identified studies to avoid duplication of data by checking authors and medical centers, examining for each one the names of all authors and the different medical centers involved. References, reviews and editorials were also screened [12]. Additional information was obtained by sending email to authors if necessary.

We didn't use strict inclusion criteria and quality score to evaluate studies, due to the lack of general agreement on the meta-analysis of observational studies [13]. All studies measuring MMPs with immunohistochemistry in patients with gynecological cancers were included.

Definitions of markers

All of the studies had their own standards in MMPs positivity. Cut-off was 10% or close to 10% in the majority of the enrolled studies. When data with this cut-off were impossible we contacted authors or calculated data based on primary information in the original article.

The main outcome of meta-analysis was MMPs-related survival (mainly focused on MMP-2 and MMP-9). There were several types of survival, such as disease free survival (DFS), cancer specific survival

(CSS), recurrence free survival (RFS), overall survival (OS) and cumulative survival (CS). We just evaluated OS which indicated the percentage of people in a study or treatment group who are alive for a given period of time after diagnosis. All studies had at least 60 months follow-up and censoring was unusual before this time point. In most studies, there were results of MMPs in stromal cells and tumor cells.

Methodological assessment

Information was carefully extracted by two of the authors (HLP and MY). Collected elements were author, publication year and country, median follow-up, FIGO stage, tumor location (ovary, uterus or cervix), MMP subtype (MMP-2 and MMP-9), definition of MMP positivity, survival type (OS, DFS, CSS, RFS and CS), cell types (stromal cells and tumor cells) and MMP antibodies. The number of patients censored alive before 60 months were also recorded. Disagreements were resolved by discussion among us. If disagreement still existed, the final decision was made by Dr. L.L.

Sometimes studies were consisted of a cohort of consecutive patients. With no quality score receiving general agreement for use in a meta-analysis, especially of observational studies, we did not weigh the quality of each study, but decisions of exclusion were always taken without knowledge of the global result of each study.

Statistics

REVMAN, version 5.1, was used for meta-analysis. Stata/SE (version 11.0) was used for Begg's and Egger's test. Survival distribution between MMP negative and MMP positive cases was significantly different with $p < 0.05$. All of the data were extracted from univariate analysis evaluating whether the results were changing gradually over time with the publication of more recent studies.

In order to evaluate the prognostic role of MMP-2 and MMP-9 in gynecological cancers, we checked the influence of MMP-2 and MMP-9 by calculating the RR and its 95% CI between negative and positive groups by a method depending on the data provided in the publication. We used Q statistics (significance for $p < 0.10$) to assess heterogeneity between studies.

Results

Studies and characteristics

Our search retrieved a total of 521 references made up by 166 "cervical cancer", 131 "endometrial cancer" and 224 "ovarian cancer". A total of 35 studies were included after screening title and abstract. Full texts were reviewed and 22 studies were excluded due to lack of survival analysis or survival data [14-16]. Finally 13 studies with 1841 patients were included in our analysis. Data on 5-year survival could be obtained from original data or survival curves (using Engauge if necessary in all of these studies). There were 4 studies for both MMP-2 and MMP-9 [17-20], 2 for MMP-9 [21,22], and 7 for MMP-2 [23-29]. Therefore, 11 studies (n=1465 patients) on MMP-2, and 6 (n=960 patients) on MMP-9 were analyzed. In insufficient studies and samples we didn't evaluate the association between MMP-2 or 9 overexpression in stromal cells and 5-year OS [18,22,24,26,27].

There were 9 studies (n=915 patients) reporting an inverse relationship between survival and MMP-2 overexpression in gynecological tumor cells, whereas 2 studies reported no such relation (n=255 patients), and one study showed favorable relation (n=295 patients). Characteristics of the 13 original studies are listed in Table 1. Cancer was located in the ovary in 1208 patients (65.6%), in the uterus in 473 patients (25.7%), and in the cervix in 160 patients (8.7%). Information on the positive cut-off ranged from 5 to 25%. FIGO stages III+IV in ovarian cancer were more frequent than in cervical and endometrial cancer.

Meta-analysis: survival at 60 months

MMP-2 overexpression in tumor cells was related to poor prognosis, leading to more deaths within 5 years. The between-study heterogeneity was significant ($p < 0.001$), so the random model was used. Mortality was 1.53-fold higher in patients whose MMP-2 in tumor cells was positive (RR 1.53; 95% CI 1.03-2.27; $p = 0.03$; Figure 1). Funnel plot was symmetrical and smaller studies (excepting no.18) gave negative results ($p = 0.721$ for Begg-Mazumdar test, $p = 0.718$ for Egger's regression test, Figure 2). MMP-9 overexpression in tumor cells was not significantly associated with survival (RR 1.26; 95% CI 0.94-1.68; $p = 0.12$; Figure 3).

The between-study heterogeneity remained significant ($p=0.008$). Mortality was 1.26-fold higher in MMP-9 positive patients. Funnel plot was asymmetrical and showed that 2 large studies [25,29] indicated negative results and conclusions were opposite to each other ($p=0.024$ with Begg-Mazumdar test; Figure 4).

Discussion

Our meta-analysis showed that MMP-2 in tumor cells, detected by immunohistochemistry, does indeed predict poor survival in gynecological cancer. However, results should be interpreted in several aspects. MMP-2 expression had a significant prognostic effect on gynecological cancers, with a RR of 1.53. Moreover, studies with larger samples showed a strong association with MMP-2 than smaller studies, but publication biases were non-significant ($p=0.721$ for Begg-Mazumdar test, $p=0.718$ for Egger's regression test, Figure 2). Although MMP-2 proved to be a potential prognostic marker, FIGO stage and histology grade may contribute to its prognostic effect too. As MMP-9 had a similar structure and function with MMP-2, we subsequently performed MMP-9 analysis. MMP-9 in tumor cells wasn't correlated with survival, yet there was a trend to increased mortal-

ity with a RR of 1.26. The negative result of MMP-9 might be caused by the limited samples and publication bias ($p=0.024$ for Begg-Mazumdar test).

In the 13 eligible studies, there was one study examining MMP-9 expression in gynecological cancers using Western blot rather than immunohistochemistry. Lengyel et al. found that high MMP-9 determined by Western blot in 92 patients had no relation with 5-year survival [21]. This finding is consistent with our meta-analysis, although a different method was undertaken. Two studies [22,25] from the same research center (Oulu University, Finland) looked for the effect of MMP-2 and MMP-9 on prognosis (disease-related survival), and suggested that MMP-2 and MMP-9 are associated with favorable prognosis. Two more studies [26,29] didn't give an explicit cut-off value of positivity; Yilmaz et al. [29] evaluated the staining of MMP-2 by scoring the intensity and distribution of positive cells which they divided into 0-4 grades. Torng et al. [26] considered stronger cytoplasmic staining of stromal cells compared to nearby tumor cells as MMP-2 positivity. However, both studies showed that MMP-2 positivity indicated poor prognosis, which is consistent with our results. MMP-2 contributes to gynecological cancers growth

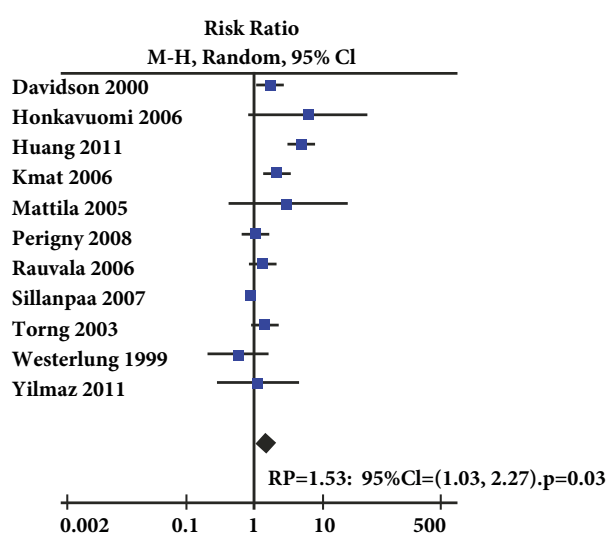


Figure 1. Meta-analysis of 11 studies evaluating the association between MMP-2 overexpression in tumor cells and the risk of death at 5 years. Each study is shown by the first author and year. CI: confidence interval.

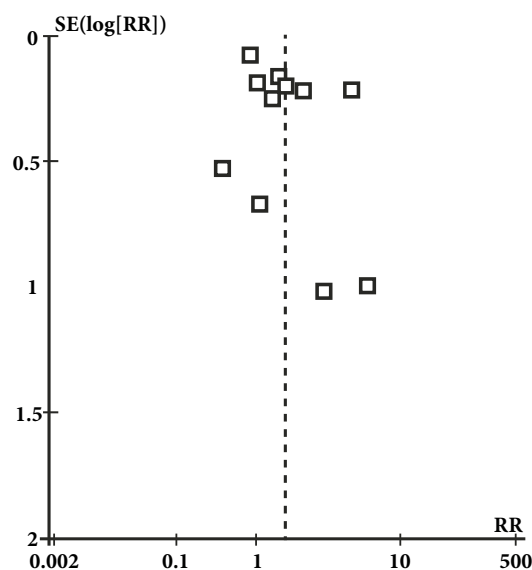


Figure 2. Funnel plot showing the relation between relative risk (RR) and standard error (log RR). The funnel plot is symmetrical.

and metastasis through several aspects. For the most, MMP-2 regulates the tumor microenvironment by directly degrading the ECM to promote cell growth and metastasis. Growing evidence demonstrates that MMP-2 proteolytically degrades the basement membrane which functions as a barrier to protect infiltration from tumor cells; this results to facilitating the tumor cells adhesion on the ECM and then invading into the peritoneal cavity, where they give rise to metastases [16,30-32].

Overexpression of MMP-2 along with surgical stage aroused our interest in the contribution of MMP-2 in metastasis [16,33,34]. The pattern of gynecological cancers' dissemination can be modeled as follows: during transformation, malignant cells are shed from the basement membrane and degrade ECM. This fact results in detachment of tumor cells, invasion into the peritoneal cavity, and development of metastases. In addition, activated MMP-2 proteolysis of the matrix including fibronectin, vitronectin and collagen I can contribute to the cancer cell adhesion and invasion [30,35-37]. These results were in line with former findings that expression of MMP-2

in metastatic locations was significantly higher than in primary cancer [38]. In conclusion, MMP-2 can regulate the gynecological cancers' growth and metastasis through distinct pathways, including promoting adhesion, invasion and angiogenesis; all these may provide an explanation for the observed modest association of MMP-2 and survival. On the other hand, the remaining members of MMP family, such as MMP-1, MMP-7, MMP-11, and MMP-13 are also considered to be associated with prognosis [39-43]. We believe that more studies are needed to evaluate the prognostic significance of these markers.

There are some clinical meanings in our meta-analysis.

1: MMP-2 positivity is an indicator of advanced stage and disease outcome

A series of studies proved that the expression of MMP-2 in gynecological tumor cells was stronger than in borderline and benign areas, and high expression of MMP-2 had close relationship with advanced tumor and metastasis [26,33,36]. Comparison of the expression and gelatinolytic activity of MMP-2 in cystadenomas, tumors of low malignant potential as well as

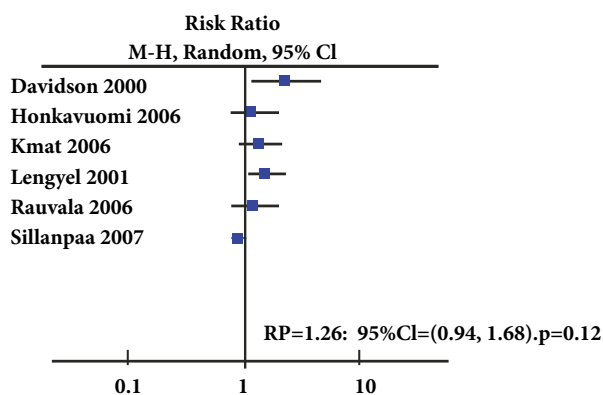


Figure 3. Meta-analysis of 6 studies evaluating the association between MMP-9 overexpression in tumor cells and the risk of death at 5 years. Each study is shown by the first author and year. CI: confidence interval.

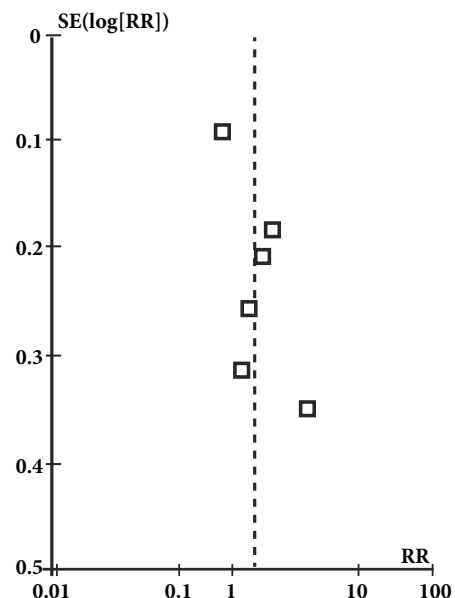


Figure 4. Funnel plot showing the relation between RR and SE (log RR). The largest study (MMP-9) has negative result. The funnel plot is asymmetrical.

stage IIIc/IV ovarian cancer and their corresponding omental metastases indicated that MMP-2 expression was strongly associated with increasing surgical stage of malignant ovarian tumors [36]. This idea has been supported by Sakata et al., who proved that increased expression of MMP-2 was related to tumor stage, which indicated that the level of MMP-2 in patients

with stage III and IV tumors is higher than in those with stage I and II tumors [17,35,45]. Tester et al. also demonstrated strong enzymatic activity of MMP-2 (62 kDa) in advanced ovarian cancer and its metastases but only seldom in benign tumors [44]. Based on this information, ovarian cancer stage and grade of malignancy can be efficiently and easily evaluated by

Table 1. Main characteristics of 13 included studies

Author (year-country) [Ref.no.]	No. of samples	Median follow-up (months)	Clinical stage (FIGO)	Tumor location	MMP subtype	Cutoff staining for MMP positivity(% cell population)	Survival analysis	Selected cells
Davidson [17] (2000-Israel)	68	70	III-IV	ovary	MMP-2 MMP-9	25	DFS OS	Tumor
Kmat [18] (2006-USA)	90	NR	I-II:24 III-IV:72	ovary	MMP-2 MMP-9	5	DFS	Tumor and stromal
Honkavuori [19] (2006-Finland)	266	NR	I+II:205 III+IV:43	uterus	MMP-2 MMP-9	10	RFS,CSS	Tumor
Rauvala [20] (2006-Sweden)	160	NR	I+II:125 III+IV:36	cervix	MMP-2 MMP-9	20	DSS	Tumor
Lengyel [21] (2001-USA)	84	55	III	ovary	MMP-9	Negative: <6U/ug	OS	Tumor
Sillanpaa [22] (2007-Finland)	292	28	I+II:125 III+IV:36	ovary	MMP-9	20	CSS,DRS,RFS	Tumor and stromal
Huang [23] (2011-China)	219	25.5	NR	ovary	MMP-2	5	OS	Tumor
Perigny [24] (2008-USA)	92	19	III	ovary	MMP-2	10	OS	Tumor and stromal
Sillanpaa [25] (2007-Finland)	295	NR	I-II:127 III-IV:168	ovary	MMP-2	10	OS,DRS,RFS	Tumor
Tornng [26] (2003-China)	35	39.9	I-II:20 III-IV:15	ovary	MMP-2	NC	CSS,DFS	Tumor and stromal
Westerlund [27] (1999-Finland)	33	35.5	I+II:12 III+IV:21	ovary	MMP-2	10	OS	Tumor and stromal
Talvensaari [28] (2005-Finland)	112	88	I+II:96 III+IV:16	uterus	MMP-2	10	OS	Tumor
Yilmaz [29] (2011-Turkey)	95	39	I:73 II+III:22	uterus	MMP-2	NC	OS	Tumor

NR: not reported, NC: not clear, CS: cumulative survival, OS: overall survival, DFS: disease free survival, RFS: recurrence free survival, CSS: cancer specific survival, DSS: disease specific survival, DRS: disease related survival

MMP-2 positive rate. A previous study of clinical and histomorphological data proved that positive staining for MMP-2 was associated with bad prognosis [38]. A large amount of research indicated that MMP-2 was significantly associated with advanced stage, higher grade, smaller tumor size at operation, and higher incidence of recurrence [21,23,25,26]. Taking these aforementioned results into account, it could be concluded that positive expression of MMP-2 plays a central role in determining disease outcome in gynecological cancers.

2: MMP can be a marker to assess timing of surgery

It is well known that histological grade is important for surgery. To further investigate the relationship between MMP-2 expression and histological grade in malignant gynecological tumors, Kamel et al. evaluated the MMP-2 expression and correlated clinical and pathological parameters, mainly surgical stage, histological grade, omental metastasis, and lymph node metastasis [16]. This study demonstrated that the expression of MMP-2 was significantly correlated with the histological grade. The authors also showed that overexpression of MMP-2 indicates poor timing of surgery. In view of these findings, histological grade can be assessed through expression of MMP-2, and then, according to the histological grade to determine the optimal operation time.

3: The present meta-analysis promotes thought on biologic therapeutic target

Signaling pathway dysfunction is a vital event in tumorigenesis that MMP-2 proteolytically activates TGF- β 1 and then TGF- β signaling pathway, the fundamental signaling pathway, which plays an important role in tumor genesis and epithelial-mesenchymal transition [45]. Several studies [46,47] have reported different types of MMPs, including MMP-2, orchestrating distinct functions (such as promoting tumor angiogenesis) on the tumors' malignant progression. MMP-2 was further proved to stimulate angiogenesis directly by releasing VEGF [37]. Blocking experiments not only confirmed the former conclusion, but also implied that inhibition of MMP-2 attenuates both angiogenesis and lymphangiogenesis, thus reducing lymph

node metastasis [48,49]. Thus, designing molecules to directly antagonize MMP-2 and indirectly block TGF- β , VEGF which mediate MMP-2 signal pathway could lead to new targeting treatments.

There are some limitations in our meta-analysis. Firstly, we attempted to minimize publication bias by improving our searching strategy. Positive results are more likely to be published while negative data may go unpublished. Secondly, although there are many studies about MMP overexpression in gynecological cancers, they often lack survival information [50,51]. Thirdly, all studies are not strictly randomized controlled trials. Although we standardized factors such as age, menopausal status, histological grade and FIGO stage, some variability was unavoidable. Between-study heterogeneity was significant in this study, and elimination of the variability (experimental design, measurements, and definition of cut-off value) was not always possible [52].

In conclusion, this meta-analysis suggested that MMP-2 overexpression is associated with poor prognosis. Our results show that MMP-9 is not correlated with prognosis in gynecological cancers, maybe owing to inadequate studies and publication biases. Future investigations and randomized controlled trials with large number of samples are needed to confirm the prognostic significance of MMP-2 and MMP-9 in patients with gynecological cancers.

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Expression of transcription factor Twist1 in bladder urothelial carcinoma and its clinical significance

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Summary

Purpose: Transcription factor Twist1 is known to play a vital role in cancer development, progression and metastasis. However, regulation mechanisms beneath Twist1 expression, as well as the correlation between its expression and bladder urothelial carcinoma (BUC), are still under investigation. Herein, we tried to investigate the expression of Twist1 in BUC specimens and non-cancerous mucosas and illustrate their relationships with clinicopathological features.

Methods: The expression of Twist1 mRNA in 42 fresh BUC specimens and 13 paired non-cancerous mucosas was detected by real-time fluorescence quantitative reverse transcription polymerase chain reaction (RFQ-RT-PCR). Immunohistochemistry (IHC) was used to detect the expression of Twist1 protein in 40 paraffin embedded BUC specimens and 14 paired non-cancerous mucosas, and their relationships with clinicopathological features.

Results: The expression levels of Twist1 mRNA in 13 paired BUC specimens were significantly lower than the non-cancerous mucosas. The positive expression rate of Twist1 protein in BUC specimens (90.0%; 36/40) was significantly higher than the non-cancerous mucosas (7.14%; 1/14). Twist1 protein was mainly distributed in the nucleus, and expressed obviously in the mesenchymal cells of several specimens (13.9%;5/36). However, expressions of Twist1 protein were not associated with TNM stage and grade. It was also shown that the expression tendency of Twist1 protein was distinct from Twist1 mRNA, and both were not correlated with age, gender, and smoking history.

Conclusion: As a probable potential biomarker for BUC, Twist1 gene may play a role as an oncogene during the tumorigenesis and development of BUC. Its abnormal protein expression may be associated with disordered regulations after transcription.

Key words: bladder cancer, gene expression, immunohistochemistry, Twist1

Introduction

As a highly conserved basic helix-loop-helix (bHLH) transcription factor, Twist1 was originally identified as a key inducer of mesoderm formation in *Drosophila*, and plays a vital role in the regulation of cell migration [1]. It's now well accepted that Twist1, which may function as an oncogene during tumorigenesis and development of cancer, promotes tumor invasion and migration through inducing the epithelial-mesenchymal transition (EMT) of cancer cells. Moreover, Twist1 is also involved with cell apoptosis, chemoresistance, angiogenesis and the generation of cancer stem cells (CSCs) [2,3]. Recently, upregulation of Twist1 protein has been reported in several cancers, such as liver [4], lung [5], stomach [6], breast [7] and bladder [8-11], and its expression levels were found to be associated with tumor progression, metastasis and poor prognosis.

Several studies have reported that expression of Twist1 protein was not correlated with its mRNA levels [12-14], a fact that was explained by post-transcriptional regulations. However, until now, the association between Twist1 protein and mRNA expression has not been established in human cancers, including BUC. Herein, we used RFQ-RT-PCR and IHC to investigate the expression of Twist1 mRNA and protein in BUC tissues and paired non-cancerous mucosas, respectively. Furthermore, we tried to describe their relationships with clinicopathological features and elucidate the association between Twist1 and BUC.

Methods

Patients

The present study was based on a consecutive series of patients undergoing surgery for BUC (transurethral resection or cystectomy) at the Department of Urology, The First Affiliated Hospital of Xiamen University, Xiamen, China. All specimens were obtained immediately after surgery, including fresh specimens and/or formalin-fixed, paraffin-embedded specimens (not all the specimens were paired). Finally, a total of 42 fresh BUC specimens and 13 paired non-cancerous mucosas (taken at least 3 cm from the outer tumor margin) were acquired, frozen in liquid nitrogen and stored at -80°C until processing. We also obtained 40 paraffin-embedded BUC specimens and 14

paired non-cancerous mucosas. All the original slides of the specimens were reviewed for histopathological staging (2002 UICC TNM classification) and grading (World Health Organization classification) by two independent pathologists. Considering that the incidence of human BUC increases especially after 50 years of age, we took the 50 years as boundary of age groups. Smoking condition of patients was evaluated by the smoking index (SI: cigarettes per day × smoking years), and divided into non-smoking group (SI < 1) and smoking group (SI ≥ 1). All patients in the study gave written informed consent whilst none had received chemoradiotherapy or immunotherapy before surgery. Clinicopathological characteristics of all the informative specimens are shown in Tables 1 and 3. The noninformative samples included unrepresentative samples or fresh samples with degraded RNA.

RNA extraction and RT-PCR

Total RNA was extracted from malignant and non-malignant samples with the RNA simple Total RNA Kit (Tiangen Inc., Beijing, China), according to the manufacturer's protocol. The RNA extracted was quantified with a Shimadzu UV-200 Spectrophotometer and stored at -80°C until processing. For reverse transcriptase RT-PCR, cDNA was synthesized from 500 ng RNA with the PrimeScript™ RT reagent Kit (Perfect Real Time, TaKaRa Code DRR037S, China).

RFQ-RT-PCR

The cDNA was then amplified by SYBR Premix Ex Taq™ (Perfect Real Time, TaKaRa Code DRR041, China), using an Light Cycler 480 analyzer (Roche, Switzerland), with the following primers: Twist1² - 5'-GGAGTCCG CAGTCTTACGAG - 3' and 5'-TCTG-GAGGACCTGGT AGAGG - 3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) - 5'-GAAGGT-GAAGGTCCGAGTC- 3' and 5' - GAAGATGGT-GATGGGATTTTC - 3'. The PCR conditions: 45 cycles of 95°C for 5 sec, 56°C for 20 sec, and 72°C for 20 sec. The initial denaturation step was performed at 95°C for 30 sec. At the end of the PCR cycles, melting curve analyses were performed to confirm the generation of the specific expected PCR product (95°C for 5 sec and 65 °C for 1 min). In the end, the relative expression of Twist1

mRNA was presented as a $2^{-\Delta Ct}$ value ($\Delta Ct = Ct_{Twist1} - Ct_{GAPDH}$), and PCR products were electrophoresed on 2% agarose gels to further identify the specificity.

Immunohistochemistry staining

IHC studies were performed using 2-step EliVision™ plus kit and diaminobenzidine (DAB) (Maixin, Fuzhou, China, Code No: KIT-9901& DAB-0031) to detect Twist1 protein expression. The paraffin slides (4µm thick) were deparaffinized and rehydrated with xylene and serial ethanol dilutions. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min, followed by antigen retrieval (boiling-treated in autoclave with 10-mmol citric buffer with pH 6.0 for 2 min). Slides were incubated for 1 h at room temperature with the Abcam mouse monoclonal Twist1 antibody (1:100) (Twist2C1a, Code No: ab50887). Phosphate buffered saline (PBS) substitute for Twist1 antibody served as negative control.

The 2-way scoring system [11] was used for analysis of Twist1 results in this study with modifications. First, we scored the staining intensity in 4 degrees: negative=0; weak=1; moderate=2; and strong=3. Then, we estimated the proportion of positive cells with the following criteria: $\leq 5\%$ =0; 6-25%, 1; 26-50%=2; and $\geq 51\%$ =3. Subsequently, the scores were added up and divided into 4 groups (≤ 1 , 2-3, 4-5, and 6) as 4 corresponding staining degrees (-, +, ++, +++).

Statistics

All statistical analyses were performed using a SPSS 13.0 software (SPSS, Chicago, IL). Normal distribution was first tested using Shapiro-Wilk test. For RFQ-RTPCR data, Mann-Whitney U test, Wilcoxon Signed Rank test (Wilcoxon test), and Kruskal Wallis test were used. The Pearson χ^2 test, correction χ^2 test and Fisher's Exact test were used for IHC data. Statistical significance was put at two-sided $p < 0.05$.

Results

Relative Twist1 mRNA expression

The results of RFQ-RTPCR showed that melting peaks of Twist1 and GAPDH were simple (Figure 1), and the expression levels of Twist1 mRNA in 13 paired BUC specimens were significantly lower than

the non-cancerous mucosas (median 1.47 and 4.05, respectively; $p < 0.01$, Wilcoxon test) (Figure 2).

As the numbers of each stage BUC specimens were small, we combined T_a and T_1 stages as the superficial bladder cancer group (T_{a-1}), and T_2 , T_3 and T_4 stages together as the invasive bladder cancer group (T_{2-4}). Twist1 mRNA expression in T_{a-1} group was about twice as much than in T_{2-4} group ($p < 0.05$, Mann-Whitney test), and its expression levels in grade (G) 3 group were significantly different from G1 and G2 ($p < 0.05$, Mann-Whitney test). However, there was no difference between G1 and G2 groups ($p > 0.05$, Mann-Whitney test). Furthermore, Twist1 mRNA expression was not correlated to age, gender and smoking history ($p > 0.05$, Mann-Whitney test) (Figure 2, Table 1).

Immunohistochemical analysis of Twist1

The positive expression rate of Twist1 protein in BUC specimens (90.0%, 36/40) was significantly higher than in the non-cancerous mucosas (7.14%;1/14) ($p < 0.001$, Pearson χ^2 test). Twist1 protein was mainly distributed in the nucleus; the percentages of BUC samples expressing the Twist1 protein either in the nucleus or in the cytoplasm were 55.6% (20/36) and 33.3% (12/36), respectively (Figure 3A,C,E). The difference between nuclear and cytoplasmic protein expression was not associated with clinicopathological features ($p > 0.05$, Fisher's Exact test, Table 2). Interestingly, Twist1 protein was expressed in the mesenchymal cells of several BUC specimens (13.9%;5/36) (Figure 3D). Unexpectedly, the expressions of Twist1 protein were not associated with TNM stage, grade, age, gender and smoking history ($p > 0.05$, Table 3).

Discussion

Two Twist genes, Twist1 (Twist) and Twist2 (Dermo-1), exist in vertebrates sharing more than 90% of identity in the carboxy-terminal domains of their proteins [15]. The human Twist1 gene located at chromosome 7p21.2 encodes the Twist1 protein consisting of 202 amino acids, which acts as a transcription factor through the pattern of dipolymer. Previously, a research [2] on breast cancer found that the transcription factor Twist1 promoted tumor invasion and metastasis through induc-

Table 1. Relationship between the mRNA expression of Twist1 and clinicopathological features

Groups	N	$2^{-\Delta\Delta ct} \times 10^{-3}$ (Percentiles)			p-value
		25%	50%	75%	
T stage					0.011
T _{a-1}	27	1.61	3.19	4.50	
T ₂₋₄	15	0.56	1.37	2.63	
Grade					0.023 ^a
1	9	1.52	1.91	4.62	
2	27	1.26	2.65	4.27	
3	6	0.37	0.85	1.48	
Age (years)					0.321
≤50	13	1.44	2.65	4.18	
>50	29	1.15	1.67	3.88	
Gender					0.666
Female	6	1.04	1.30	5.87	
Male	36	1.39	2.12	3.86	
Cigarette smoking					0.875
SI<1	11	1.16	1.68	5.30	
SI≥1	31	1.37	1.91	3.85	

^aKruskal Wallis test. Multiple comparisons (Wilcoxon test: G1 and G2, p=0.898; G1 and G3, p=0.010; G2 and G3, p=0.012 (2-tailed). SI: smoking index

tion of EMT in cancer cells. Loss of E-cadherin appears to be critical to an EMT. Similar with Snail or Slug, Twist1 inhibited E-cadherin expression at the transcriptional level through binding to E-boxes elements of the E-cadherin promoter. Twist1 mRNA and protein were both increased in hepatocellular carcinoma as compared with non-cancerous tissues, and the upregulated Twist1 protein was associated with metastasis and poor prognosis [4]. Recently, an article about 151 colorectal cancer cases demonstrated that Twist1 expression was restricted to tumor tissues and correlated with lymph node metastasis and overall survival (OS) [16].

In our study, Twist1 protein was significantly up-regulated in BUC tissues compared to non-cancerous mucosas, but without any correlation with TNM stage or grade of malignancy, indicating its potential importance in the tumorigenesis and development of BUC, especially in early stage. In addition, we found Twist1 protein was mainly expressed in cell nuclei without any association with clinicopathologic data, suggesting that Twist1 protein may regulate the cellular life activities and promote cancer development

through pathways of nuclear translocation. A previous study [11] with 164 bladder cancer tissues, 37 nonmalignant bladder tissues and 25 matched lymph node metastatic lesions, also showed that the positive rate of Twist1 protein was significantly elevated in cancer tissues (>90%) compared with nonmalignant tissues (about 20%). However, the authors found that Twist1 protein expression was found mainly in the cytoplasm and was associated with cancer progression. Yet, in recent years, several studies on bladder cancer found that the positive rate of Twist1 protein was about only 40% in tumor tissues [8-10], and its expression was associated with smoking history [10]. Particularly, Gort et al. also described nuclear Twist1 staining in malignant cells, and found no correlation between Twist1 expression and clinicopathologic data [12]. Certainly, more studies with larger sample size are needed to confirm our present findings.

Simultaneously, we found strong positive nuclear expression of Twist1 protein in the stromal compartment of several BUC tissues, where there exist two cell populations (epithelial-mesenchymal trans-

formed tumor cells and stromal fibroblastic cells). Twist1 expression might be activated in these cells due to growth factors produced by the tumor. However, expression of Twist1 protein in the stromal compartment was not associated with prognosis [7]. In another study, Twist1 expression was especially observed in gastric cancer-associated fibroblasts, and was associated with disease progression and poor patient survival [17]. Obviously, more clinical studies and follow up data are needed to validate the expression sites of Twist1 protein, as well as its correlation with tumor progression and patient prognosis.

We found that Twist1 mRNA expression in non-cancerous bladder mucosas was significantly higher than paired cancer tissues, and its expression in T_{a-1} stage group was higher than in T₂₋₄ stage group. These findings implied that the pre-transcriptional regulations, such as DNA promoter methylation, might be involved in suppressing Twist1 mRNA expression. In accordance with other studies [12,18], a detection

base on 91 BUC tissues and 39 non-cancerous bladder mucosas found that the promoter methylation level of Twist1 in cancer tissues was significantly higher than in non-cancerous tissues [19]. Furthermore, the hypermethylation frequencies of Twist1 in cervical carcinoma increased progressively along with tumorigenesis and cancer development [18]. Although regulations before transcription seem to explain our results, there was no evidence to support relevance between Twist1 promoter methylation and mRNA expression until now. Gort et al. considered other signal pathways involved in the regulations [12]. Indeed, we need more basic research with *in vitro* models to further elucidate the intrinsic regulation mechanisms of Twist1 mRNA.

In this study, the trend of Twist1 protein expression was discordant with mRNA expression, suggesting that Twist1 protein might be regulated mainly through post-transcriptional actions. Similarly, no correlation between Twist1 protein and mRNA ex-

Table 2. Correlation between Twist1 protein expression positions and clinicopathological features

Groups	Twist1 expression positions			p-value
	Positive Num ^a	N1 ^a +B/2 ^b (%)	N2 ^a +B/2 ^b (%)	
T stage				0.471
T _{a-1}	24	15+1 (66.7)	7+1 (33.3)	
T ₂₋₄	12	5+1 (50)	5+1 (50)	
Grade				0.054
1	7	5 (71.4)	2 (28.6)	
2	23	9+2 (47.8)	10+2 (52.2)	
3	6	6 (100)	0 (0)	
Age (years)				0.706
≤50	10	7 (70)	3 (30)	
>50	26	13+2 (57.7)	9+2 (42.3)	
Gender				1.000
Female	5	3 (60)	2 (40)	
Male	31	17+2 (61.3)	10+2 (38.7)	
Cigarette smoking				0.441
Yes	28	14+2 (57.1)	10+2 (42.9)	
No	8	6 (75)	2 (25)	

a: Positive Num means the number of positive specimens; b: N1 and N2 denote the numbers of samples expressing Twist1 protein either in the nucleus or in the cytoplasm, respectively; B: signifies the number of samples expressing Twist1 protein both in the nucleus and cytoplasm

pression was found in a breast cancer research [12], and difference in Twist1 protein expressions was also explained by post-transcriptional regulation. In contrast, a previous detection consisting of 8 paired BUC tissues and normal mucosas, found that the positive rate of Twist1 mRNA in malignant tissues (100%) was significantly higher than in paired normal mucosas (12.5%), in accordance with its protein expression [11]. Nevertheless, review of the literature revealed consensus that Twist1 protein expression was mainly associated with post-transcriptional regulations (post-translational and translational modification). Many studies found that the phosphorylation degrees of Twist1 protein in tumors positively correlated with its expression levels, suggesting that phosphorylation of Twist1 protein stabilized its expression [13,14,20,21]. On the other hand, a very recent study found several putative regulatory elements at the Twist1 3'UTR (untranslated regions), consisted of miRNA target sites and two cytoplasmic polyad-

enylation elements, which could restrain the Twist1 protein translation through specifically binding with miR-580 etc [22]. Although these findings could partly explain our results, more studies are needed to clarify the relationship between Twist1 mRNA and protein expression.

In conclusion, our study demonstrated that Twist1 protein expression in BUC specimens was significantly higher than the adjacent non-cancerous mucosas and was not associated with clinicopathological features. Moreover, Twist1 protein was mainly distributed in the nucleus and expressed in the mesenchymal cells of several malignant specimens; Twist1 mRNA expression in malignant specimens were significantly lower than in paired non-cancerous mucosas (discordance with Twist1 protein expression), and correlated with grade and TNM stage.

Finally, we conclude that Twist1 may play an oncogenic role during tumorigenesis and development of BUC, and its abnormal protein expression might

Table 3. Relationship between the expression of Twist1 protein and clinicopathological features

Groups	N	-	+	++	+++	%	p-value
BUC	40	4	27	7	2	90.0	0.000 ^a
Non-cancerous	14	13	1	-	-	7.14	
Tstage							1.000 ^b
T _{a-1}	27	3	18	5	1	88.9	
T ₂₋₄	13	1	9	2	1	92.3	
Grade							0.673 ^c
1	8	1	5	2	-	85.5	
2	26	3	18	4	1	88.5	
3	6	-	4	1	1	100.0	
Age (years)							0.730 ^d
≤50	12	2	7	3	-	83.3	
>50	28	2	20	4	2	92.9	
Gender							0.493 ^e
Female	6	1	4	1	-	83.3	
Male	34	3	23	6	2	91.2	
Cigarette smoking							0.543 ^f
SI≥1	30	2	23	4	1	93.3	
SI<1	10	2	4	3	1	80.0	

a,c:Pearson's χ^2 test; b,d,f: Correction χ^2 test; e:Fisher's exact test; SI: smoking index; BUC: bladder urothelial carcinoma

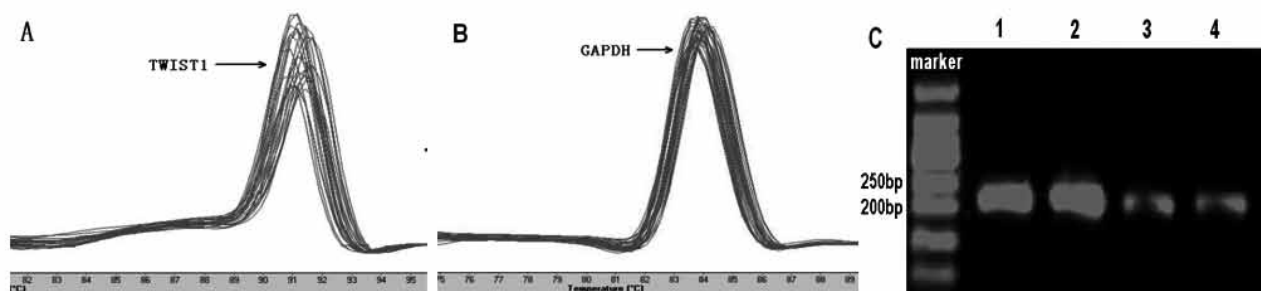


Figure 1. Melting peaks of RFQ-RT-PCR products and DNA gel electrophoresis. A, B: Melting peaks of target gene Twist1 and reference gene GAPDH, respectively; C: DNA gel electrophoresis of Twist1 (201bp); 1-4 indicate different samples.

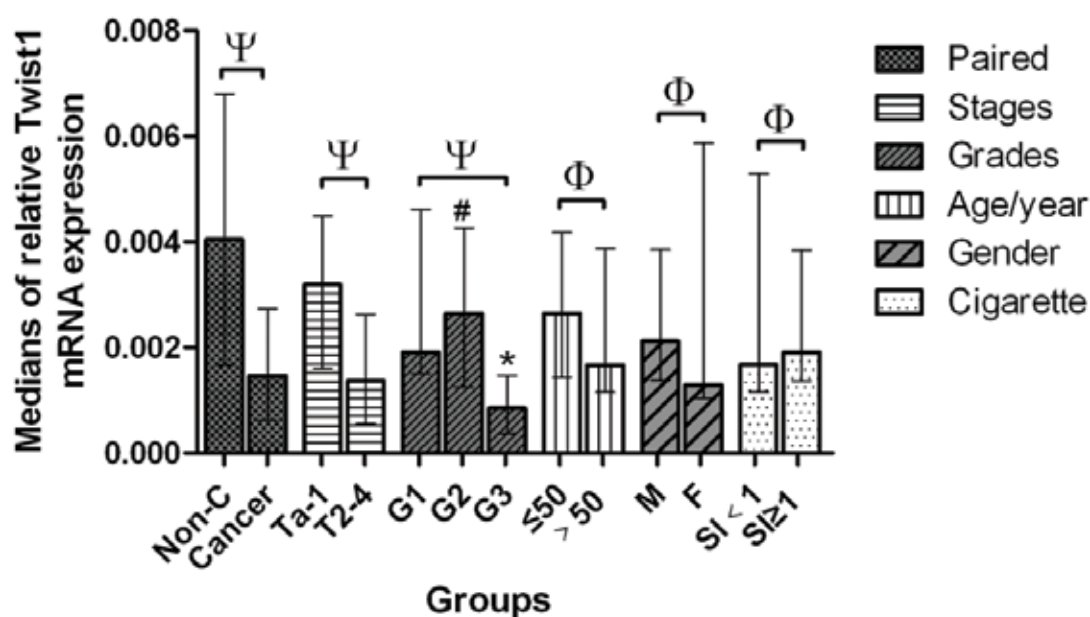


Figure 2. Medians and interquartile ranges of relative Twist1 mRNA expression in different groups. Non-C: paired non-cancerous mucosa; Ψ: $p < 0.05$; Φ: $p > 0.05$. *: G3 vs G1, $p < 0.05$; G3 vs G2, $p < 0.05$; #: G1 vs G2, $p > 0.05$.

be associated with the disordered regulations after transcription. Twist1 may be utilized as a new potential biomarker for BUC. Undoubtedly, in order to further verify the correlations between Twist1 expression and clinicopathological features, multicentric clinical researches and followup studies are required. Meanwhile, more fundamental research are needed to further elucidate the regulatory mechanisms be-

neath Twist1 expression, as well as Twist1-related signal pathways involved in BUC.

Acknowledgements

This research was supported by the Medical Center Construction Foundation of Xiamen, as well as the Science and Technology Office Funds for Key Project of Fujian Province, China (No.2009D023). We

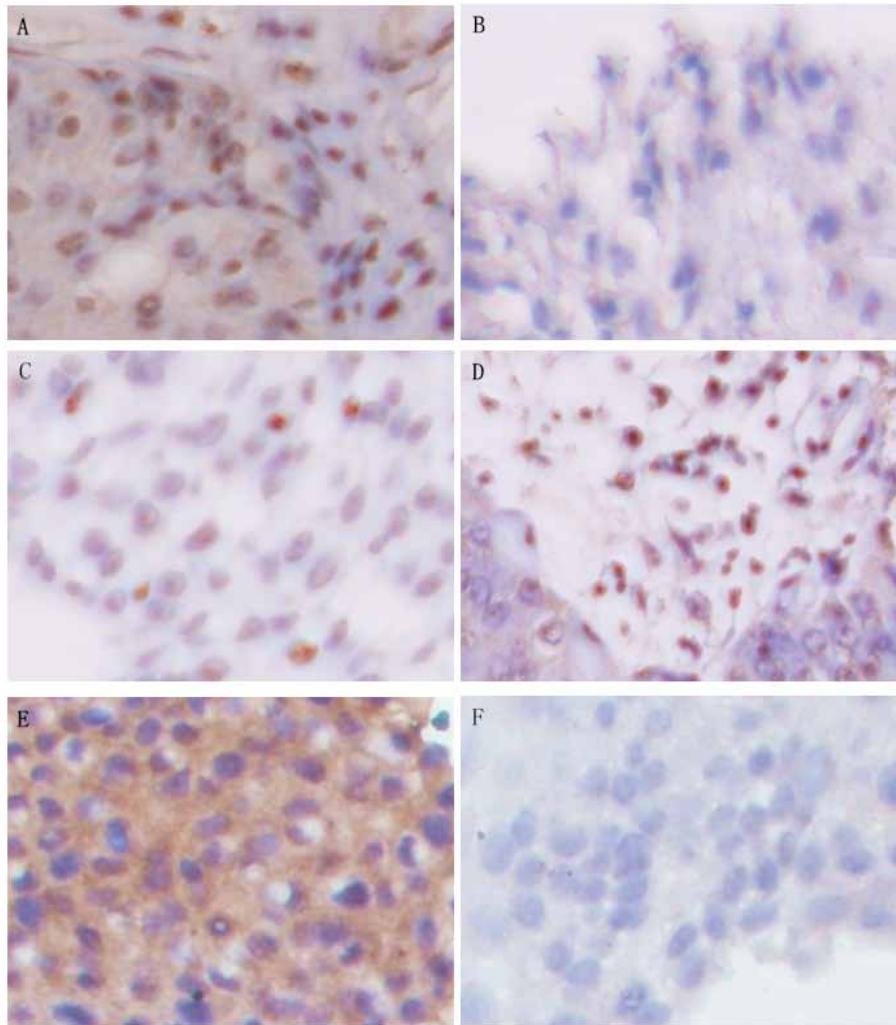


Figure 3. A,B,C,D,E,F: Expression of Twist1 protein in BUC tissues and paired non-cancerous mucosa (EliVision™, ×400).

A&B: Positive and negative expression of Twist1 protein in a BUC tissue (G2,T1) and paired normal mucous membrane, respectively; C: Twist1 protein expressed in a BUC tissue (G2,T1); D: Strong positive nuclear expression of Twist1 protein in the mesenchymal cells of a BUC tissue (G2,T1); E: Strong positive cytoplasmic expression of Twist1 protein in a BUC tissue (G2,T2b); F: Negative expression of Twist1 protein in a BUC tissue (G2, T2a).

thank Dr. Haiping Zhang and Dr. Meihua Ye for review of paraffin-embedded sections.

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Small cell carcinoma of the bladder: A search of the current literature

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Summary

Purpose: Small cell carcinoma of the urinary bladder (SCC-BL) is an extremely rare malignancy, accounting for < 1% of all bladder tumors. Its prognosis is very poor because of its highly aggressive behavior and high metastatic potential. This study aimed to update the management and outcome of SCC-BL by searching the relevant international literature.

Methods: Relevant studies were identified by searching MEDLINE and the Cochrane Central Register of Controlled Trials using a combination of terms such as small cell carcinoma, bladder cancer, therapeutic approach, radical cystectomy, radiation therapy and chemotherapy. Additional papers were identified from reviewing references of relevant articles.

Results: Previously published series have shown that SCC-BL has a significant male predominance, occurs mainly during the 7th and 8th decade of life and macroscopic hematuria is the most common presenting symptom. According to the most important studies, cystectomy alone seems not to be efficient enough for the management of the disease. On the other hand, radiation therapy when combined with chemotherapy is highly effective with increased survival rates.

Conclusion: Poor prognosis and rarity render disease management complicated. A definitive treatment is not yet established but combined therapy with systemic platinum-based chemotherapy and adjuvant local radiotherapy seems to be the most effective therapeutic approach for limited-stage SCC-BL. Further research is required in order to clarify whether prophylactic cranial irradiation (PCI) should be performed on a regular basis.

Key words: bladder cancer, chemotherapy, radiation therapy, small cell carcinoma

Introduction

Small cell carcinoma (SCC) comprises a fifth of lung cancers [1] but has also been described in extrapulmonary sites such as esophagus, breast, larynx and bladder. Reported extrapulmonary sites in the literature are summarized in Table 1.

SCC-BL, histologically identical to small cell lung carcinoma (SCLC), is an extremely rare malignancy that accounts for < 1% of all bladder tumors. Incidence rates of SCC-BL range from 0.48 to 1% according to some important studies shown in Table 2.

The first case [14] was reported in 1981 and since then about 1000 cases have been published in small series and case reports. The prognosis is very poor as SCC-BL is associated with a highly aggressive behavior and high metastatic potential. The 5-year overall survival rates depend on the stage of disease at the time of the diagnosis [15] and the kind of treatment.

Herein, a search of the current literature on the management and outcome of SCC-BL was attempted and presented.

Methods

Identification of eligible studies

We searched MEDLINE and the Cochrane Central Register of Controlled Trials (last search on May 2012) using combinations of terms, such as: small cell carcinoma, bladder carcinoma, therapeutic approach, radical cystectomy, radiation therapy and chemotherapy. We considered all English-written metaanalyses, randomized controlled trials and research trials providing evidence about the therapeutic interventions in SCC-BL and future directions of ongoing research.

Data extraction

Information was extracted from each eligible study. The data recorded included author's name, year of publication, number of patients included in the study, combination(s) of treatment(s) used, doses of radiation therapy, disease free survival, median time to progression, median and overall survival.

Results

Bladder cancer accounted for 5% of all newly diagnosed cancers and was the second most common urologic ma-

lignancy. The incidence rates in Western countries were the highest. Transitional cell carcinomas represented the majority (> 90%) of all bladder cancers. Pure squamous cell carcinomas accounted for 5 to 8%, pure adenocarcinomas for 1 to 2% and the rare extra pulmonary SCC-BL accounted for < 1% (Table 2).

Some of the most known causes which seem to be associated to bladder cancer were cigarette smoking, exposure to industrial chemicals, obstructive uropathy, fertilizers, radiation exposure, genetic predisposition and probably coffee consumption [11,16].

Previously published series have shown that SCC-BL had a significant male predominance with a male to female ratio between 2:1 – 16:1 [8,12,15,22-26]. SCC-BL occurred mainly during the 7th and 8th decade of life [8-10,12,15,16,18-22,27-30]. Mean age at the time of diagnosis was usually 67 years. Macroscopic hematuria was the most common presenting symptom (>63%) at the time of diagnosis [8,9,11,12,15,16,18,19,21,22,24,31], followed by dysuria and irritative symptoms. Cushing's syndrome [12,32] and hypercalcemia [33] were also reported in rare cases.

SCC-BL was usually diagnosed as locally advanced disease, presented on MRI or CT scan as a locally invasive mass (Figure 1). Additionally, SCC-BL was characterized by a high metastatic potential involving parailiac and paraaortic lymph nodes, liver, vertebral and costal bones, abdominal cavity and brain [8,12,16].

Histologically SCC-BL was identical to SCLC, composed of small round tumor cells with sparse cytoplasm, round to oval nucleus and numerous mitotic figures (Figure 2). Specimens were obtained by cystoscopy and transurethral biopsy [8]. Immunohistochemistry was also very important for the diagnosis of SCC-BL, showing positive immunoreactivity to neuroendocrine markers including NSE (neuron specific enolase), synaptophysin, serotonin, chromogranin and epithelial markers including cytokeratin and CAM 5,2 [8]. Low incidence rates and aggressive clinical course of SCC-BL render disease management complicated. Cystectomy alone as treatment of SCC-BL seemed not to be efficient, especially for patients diagnosed with advanced disease stages [13,18,28,34].

Table 1. Reported small cell carcinoma in extra-pulmonary sites

<i>First author</i>	<i>Year of publication</i>	<i>Extrapulmonary sites reported</i>
Matsusaka [2]	1975	Stomach
Wade [3]	1983	Breast
Capella [4]	1984	Kidney
Galanis [5]	1997	GI: pancreas, esophagus, larynx, salivary glands; GU: penis, bladder, prostate; GYN: endometrium, ovary, cervix, lymph nodes; submandibular lymph nodes, inguinal lymph nodes, thymus
Ibrahim [6]	1984	Head/ neck, esophagus, stomach
Abbas [7]	1995	Pharynx, larynx, esophagus, pancreas, vagina, uterus, skin, breast, cervix, kidney, prostate, bladder

GI: gastrointestinal, GU: genitourinary, GYN: gynecological

Table 2. Incidence rates of small cell carcinoma of the bladder

<i>First author</i>	<i>Year of publication</i>	<i>Number of patients</i>	<i>Frequency of SCC-BL, %</i>
Blomjous [8]	1989	18	0.48
Lopez [9]	1994	6	1
Holmang [10]	1995	25	0.70
Lohrisch [11]	1999	14	0.35
Choong [12]	2005	44	0.5
Quek [13]	2005	25	1

SCC-BL: small cell carcinoma of the bladder

Cheng et al. [15] reported on 64 patients. The authors found that there was no significant 5-year overall survival difference between the 38 patients who had undergone cystectomy alone and those who hadn't undergone cystectomy and received combined modality treatments ($p=0.65$). The exact 5-year disease-free survival rates were 16 and 18%, respectively. Grignon et al. [18] reported on 22 patients; 5 of these underwent radical cystectomy and received adjuvant chemotherapy. In these patients overall survival was higher but not statistically significant in comparison with the other patients who hadn't received adjuvant chemotherapy ($p>0.10$). Surgery alone seems to be effective only for early disease stages, however diagnosis of SCC-BL is rarely performed at early stages [10]. Choong et al. [12] reported on 44 patients and the 5-year overall survival rates for patients with stage II, III, IV disease were 63.6, 15.4 and 10.5%, respectively. The authors proposed that all patients with limited disease should undergo radical cystectomy and adjuvant treatment should be considered for patients with stage III and IV disease.

The most commonly used chemotherapy regimen was etoposide and cisplatin (EP). Holmang et al. [10] reported on 25 cases, concluding that limited stage SCC-BL can be cured by partial or radical cystectomy combined with radiotherapy. Total doses of 51 and 67 Gy had been delivered to long-term survivors in that series.

The most important studies are summarized in Table 3. Based on their results, one can conclude that chemotherapy is crucial for the treatment of SCC-BL. Neoadjuvant chemotherapy followed by surgery is proven to improve the 5-year disease-free survival. Siefker-Radtke et al. [29] reported on 46 patients. Twenty-one patients received neoadjuvant chemotherapy before radical cystectomy and 78% achieved a 5-year overall survival which was much higher compared to 36% 5-year overall survival of 25 patients who had undergone radical cystectomy alone. Moreover, the majority of the studies showed that adjuvant chemotherapy is associated with increased 5-year overall survival. Blomjous et al. [8] reported on 18 cases. Five patients who received chemotherapy

Table 3. Eligible studies for small cell carcinoma of the bladder

<i>First author [Ref]</i>	<i>Year of pub- lication</i>	<i>Number of patients</i>	<i>Treatment</i>	<i>2- year overall survival %</i>	<i>5- year overall survival %</i>	<i>Overall survival (months)</i>
Blomjous [8]	1989	18	Chemotherapy (5pts)	60	NR	NR
			No chemotherapy (13pts)	15.5	NR	NR
Lopez [9]	1994	6	Cystectomy alone (4pts)	NR	NR	NR
			Cystectomy-radiotherapy (1pt)	NR	NR	5-25
			TURB – radiotherapy (1pt)	NR	NR	NR
Holmang [10]	1995	25	Radical cystectomy- radiotherapy (18pts)	28	28	0
			Chemotherapy (2pts)	0	0	7.15
			No therapy (5pts)	0	0	-
Lohrisch [11]	1999	14	Chemotherapy-radiotherapy (8pts)	88	NR	NR
			Chemotherapy-radical cystec- tomy (1pt)	70	44	
			Radiotherapy (2pts)	0	0	15.11
			No therapy (2pts)	0	NR	8.2
Siefker- Radke [29]	2004	46	Neoadjuvant chemotherapy – radiotherapy (21pts)	NR	78	NR
			Radical cystectomy alone (25pts)	NR	36	NR
Abrahams [16]	2005	51	Cystectomy (12pts)	NR	40	23
			Chemotherapy (9pts)	NR	NR	NR
			Neoadjuvant chemotherapy- radical cystectomy (9pts)	NR	NR	NR
Choong [12]	2005	44	Radical cystectomy (17pts)	NR	NR	NR
			Partial cystectomy (5pts)	NR	NR	NR
			Radical cystectomy-chemothera- py (12pts)	NR	NR	NR
			Chemotherapy (5pts)	NR	NR	NR
Bex [27]	2005	25	Chemotherapy (13pts)	NR	NR	15
			No chemotherapy (12pts)	NR	NR	4
Mukesh [36]	2008	20	Chemotherapy (13pts)	NR	NR	33
			No chemotherapy (7pts)	NR	NR	3
Siefker- Radke [25]	2009	30	Resectable SCC-BL (18pts)	NR	NR	58
			Non resectable SCC-BL (12pts)	NR	NR	13.3
Bex [17]	2009	17	Sequential chemoradiotherapy (17pts)	56	36	NR
Bex [26]	2010	51	Limited disease	NR	NR	35
			Extensive disease	NR	NR	6

Pts: patients, NR: not reported

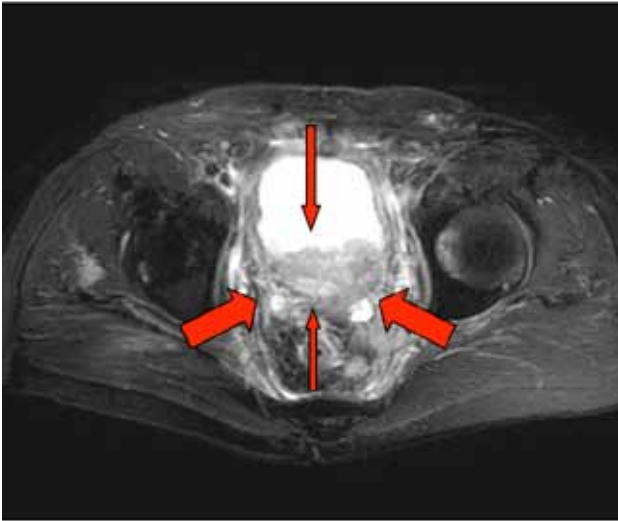


Figure 1. MRI of abdomen and pelvis showing small cell carcinoma of the bladder as a locally invasive mass (arrows).

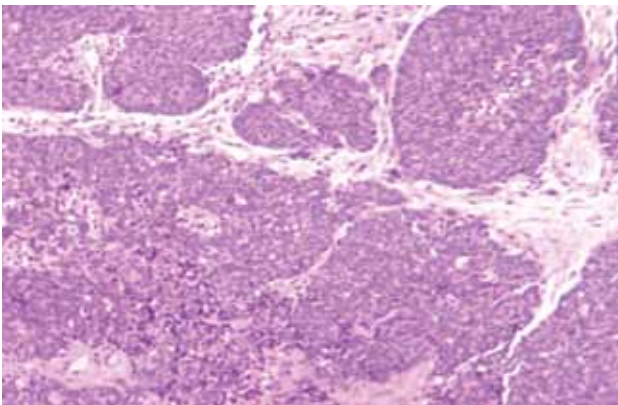


Figure 2. Immunohistochemistry of small cell carcinoma of the bladder showing small cells with sparse cytoplasm and hyperchromatic nuclei (hematoxylin and eosin x20).

(doxorubicin, cyclophosphamide and methotrexate-cisplatin) had prolonged overall survival periods (15-38 months). The authors suggested that chemotherapy may offer considerable benefit. Bex et al. [27] reported on 25 patients, 13 of whom received platinum-based chemotherapy; 5 of these patients had undergone complete transurethral resection of the bladder (TURB) before chemotherapy. Overall survival was 15 months vs. 4 months of those without chemotherapy ($p=0.028$).

Due to the similarities of histological and clinical course of SCC-BL with SCLC, combined chemoradiotherapy is preferred as the main treatment for

SCC-BL [8,11,18,34,35]. Lohrisch et al. [11] reported on 14 patients (71% underwent surgery) and observed 70% 2-year and 44% 5-year overall survival in 10 patients who were treated with chemotherapy and local radiotherapy. Common chemotherapy regimens used were etoposide and cisplatin (EP) and cisplatin, doxorubicin, vincristine and etoposide (PAVE). Total radiotherapy doses ranged from 3500 to 6400 cGy. Studies have also shown that radiation therapy when combined with chemotherapy was highly effective compared to radiation therapy alone [10,17]. Bex A et al. [17] reported on 17 patients, all treated with sequential chemoradiotherapy and concluded that the clinical results of this approach were comparable to a series of SCC-BL treated with cystectomy and adjuvant chemotherapy. All patients received platinum-based chemotherapy and radiotherapy with mean total dose of 60 Gy.

Several studies have reported brain relapses in patients with SCC-BL [18,19,35]. However, the necessity of PCI performed regularly in patients with SCC-BL, like in patients with SCLC, is still a matter of debate.

Discussion

According to the most important studies summarized in Table 3, prognosis is very poor for patients with SCC-BL. Prognosis is strongly related to the stage of the disease at the time of diagnosis [15]. Overall survival was higher in patients diagnosed with limited disease [26]. Additionally performance status and the level of serum lactate dehydrogenase (LDH) are factors with prognostic significance [11].

Clinical course is very aggressive and renders disease management complicated. There is not yet a definitive treatment but it seems that systemic platinum-based chemotherapy, especially when combined with local radiotherapy, is highly effective. Overall survival was higher in patients who were treated with adjuvant systemic chemotherapy in comparison with patients who had undergone cystectomy alone as well as patients who had received chemotherapy or radiotherapy alone.

The most commonly used regimen was cisplatin and etoposide in analogy to SCLC. Etoposide was administered at 100mg/m intravenously for 3 consecutive days repeated every 3 weeks. Cisplatin was given

at 80-100mg/ m on day 1.

SCC-BL is a very rare malignancy. Pathogenesis of the disease remains still unclarified. Several hypothesis were proposed to explain the origin of SCC-BL. Cramer et al. [14] suggested that SCC-BL arises from metaplastic changes of urothelium. Neuroendocrine cells had been documented previously in the urinary bladder [22]. Ali et al. [22] proposed the most important hypothesis that SCC-BL arises from malignant transformation of bladder neuroendocrine cells.

Brain relapses in patients with SCC-BL are documented in the literature [18,29,35] although in lower rates compared to those of SCLC. Therefore there is not yet evidence that PCI should be performed regularly in patients with SCC-BL.

Conclusion

SCC-BL is a very rare and extremely aggressive malignancy. At the time of diagnosis the disease is usually at advanced stage (pelvic lymph nodes or distant metastasis). Poor prognosis and its rarity render management difficult. No definitive treatment is yet established, but combined therapy with systemic platinum-based chemotherapy and local radiotherapy with preservation of the bladder seems to be the most efficient therapeutic approach for patients with limited disease.

Further research is required in order to clarify whether PCI should be performed on a regular basis, as it is a common practice in the management of SCLC.

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Association of Cyclin D1 A870G polymorphism with two malignancies: Acute lymphoblastic leukemia and breast cancer

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Summary

Purpose: To study the association between Cyclin D1 (CCND1) polymorphic variants and acute lymphoblastic leukemia (ALL) and breast cancer cases and the possibility of having different (CCND1) polymorphic variants in the development of ALL and breast cancer. In addition, to study the association between the different CCND1 polymorphic variants and the response to induction chemotherapy in ALL cases and clinicobiological parameters in breast cancer.

Methods: We evaluated the association of CCND1 G870A polymorphism with ALL risk in 25 ALL patients and 15 healthy controls and with breast cancer risk in 30 newly diagnosed breast cancer female patients and in 25 healthy controls. Restriction Fragment Length Polymorphism (RFLP) polymerase chain reaction (PCR) was used for analysis of G870A polymorphism of CCND1 on anticoagulated whole blood of both the ALL and breast cancer cases and control groups.

Results: The frequency of the AA genotype was significantly increased in the ALL cases while GG genotype was significantly increased in the control group. Furthermore, there was a highly statistically significant association between the A allele in the homozygous state AA and the ALL cases. Furthermore, there was a positive risk of developing ALL when having the AA genotype and the results were highly significant for AA genotype compared to GG genotype. For breast cancer, the results revealed that there was a positive risk association for those carrying the CCND1 A allele in the development of breast cancer.

Conclusion: Homozygosity for CCND1 A allele was associated with ALL patients and was a risk factor for ALL development, while the presence of the A allele, whether in homozygous or heterozygous state was associated with breast cancer cases and was a risk for breast cancer. Homozygosity for CCND1 G allele was associated with the control group.

Key words: acute lymphoblastic leukemia, breast cancer, cyclin D1, RFLP-PCR

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Received: 06/04/2012; Accepted: 05/05/2012

Introduction

Cancer continues to be a worldwide killer, despite the enormous amount of research and rapid developments seen during the past decade. According to recent statistics, cancer accounts for about 23% of the total deaths in the USA and is the second most common cause of death after cardiovascular diseases [1].

Molecular epidemiology is an emerging new field that combines highly sensitive molecular techniques for detecting early damages associated with cancer. For this purpose, it is necessary to use biological markers as indicators signaling events in biological systems or samples [2]. One of the main biological markers is the marker of susceptibility, especially in preexisting inherited genetic defects that increase the risk of cancer [3].

Single nucleotide polymorphism (SNP) was defined by genome-wide association studies as a DNA sequence variant. Many of SNPs were strongly associated with diseases in large case-control studies [4].

Cyclin-dependent kinases (CDKs) are protein kinases involved in critical cellular processes, such as cell cycle or transcription, whose activities requires association with specific Cyclin subunits. Cyclins are proteins which act as key controlling elements of the eukaryotic cell cycle and function as allosteric regulatory subunits for the CDKs catalytic subunit [5].

The importance of Cyclin-CDK complexes in cell proliferation is underscored by the fact that deregulation in the function of these complexes is found in virtually the whole spectrum of human tumors and this comes from the fact that tumor-associated alterations in Cyclins help to sustain proliferation independent of external mitogenic or anti-mitogenic signals [6].

CCND1 is a 35-kDa protein which is encoded by 5 exons situated at the region of chromosome band 11q13. In the amino terminus of CCND1 appears a motif Leu - X - Cys - X - Glu (X represents any aminoacid) where pRB (retinoblastoma protein) pocket domain binds. The carboxy terminus inhibits myogenic helix loop helix (HLH) protein function. HLH protein's main action is to remove cells from the cell cycle (halt proliferation), so its inhibition by CCND1 leads the cell to G1 phase of the cell cycle [6].

CCND1 is overexpressed in several human tu-

mors. Chromosomal translocations, gene amplification and disruption of normal intercellular trafficking and proteolysis are the procedures which lead to accumulation of CCND1 in tumor cell nuclei and eventually to CCND1 overexpression in many tumors [6].

CCND1 is the major D Cyclin in most cell types. All 3 Cyclin D molecules act in late G phase, just before entry into S phase. Many tumors have high CCND1 levels without amplification or mutation of the CCND1 structural gene [7]. Unlike other Cyclins, D-Cyclins are strongly dependent on extracellular mitogenic stimuli [8]. Due to this property they are regarded as mitogenic sensors that relay signals from the extracellular environment to the core cell cycle machinery [9].

CCND1 expression and accumulation are induced by growth factors and occur at multiple levels including increased transcription, translation, and protein stability. Regulation is mediated primarily through Ras (rat sarcoma) signaling pathways [10].

Following its induction by mitogenic growth factors, newly synthesized CCND1 associates with CDK4; the predominant function of the D1/CDK4 enzyme involves the phosphorylation-dependent inactivation of the retinoblastoma protein [11].

Although intragenic somatic mutation of CCND1 in human disease is rare, CCND1 gene translocation, amplification and/or overexpression are frequent events in selected tumor types. A polymorphism of CCND1 that occurs in a splice donor site has been epidemiologically linked to increased cancer risk or poor prognosis in a number of tumor types. Recent functional analyses have revealed that protein product of an alternately spliced transcript, CCND1b, harbors overlapping but distinct functions as compared to full length CCND1 [12].

Acute leukemia is the major pediatric cancer affecting between 30-45 per 1,000,000 children each year [13]. Current protocols for diagnosing and treating ALL have achieved overall cure rates, defined as the absence of disease for at least 10 years, of more than 80% in children, while adults with ALL still have a relatively poor prognosis [14].

The causes of the vast majority of ALL cases are unknown. Among childhood, only ionizing radiation

and certain genetic disorders are known risk factors. Many other risk factors have been suggested but remain under investigation, such as exposure to pesticides, automobile exhaust, certain chemicals such as benzene, non ionizing radiation (e.g., magnetic fields), parental exposures (e.g., cigarette smoking, alcohol consumption and use of some pharmaceuticals), and even parental consumption of certain dietary constituents [15].

The genetic candidates that have been evaluated as susceptibility genes for childhood ALL to date can be broadly delineated into those coding for carcinogen metabolism enzymes, folate metabolism enzymes, DNA repair proteins, and others [13].

Breast cancer is a heterogeneous disease that encompasses several distinct entities with remarkably different biological characteristics and clinical behaviors. Among women, breast cancer remains the most commonly diagnosed cancer. Genetic risk factors contribute to about 5-10% of all cases, 90-95% of them result from somatic mutation and about 5-10% are inherited as a result of germ line mutation in autosomal dominant breast cancer susceptibility genes [16].

The aim of the present work was to study the association between CCND1 genetic polymorphism and the risk of different types of human cancers as compared to control groups. This study investigated the associated risk of CCND1 polymorphism with the development of ALL and breast cancer.

Methods

Patients

This study was carried out on 25 patients with newly diagnosed ALL, who presented to the Hematology Department, Medical Research Institute, University of Alexandria and Elshatby Children hospital, University of Alexandria and 30 patients newly diagnosed with breast cancer, who presented to the Oncology Department, Medical Research Institute, University of Alexandria and National Cancer Institute Out-patient Clinic from January 2010 to June 2011, after obtaining oral informed consent. The diagnosis of ALL was based on complete blood count, bone marrow aspiration and immunophenotyping, while the diagnosis of breast cancer was based on initial mam-

mographic screening and biopsy. The study included age and sex matched control groups, i.e. 15 healthy children for the ALL group and 25 healthy females for the breast cancer group.

All patients were subjected to careful history taking, thorough physical examination, complete blood count, liver and renal function tests, plain chest X-ray and abdominal ultrasound. For ALL patients: bone marrow examination and immunophenotyping by flow cytometry using B and T cell markers (DAKO, Denmark) were performed. For breast cancer patients: mammography and immunohistochemical (IHC) staining for estrogen and progesterone receptors status were carried out on formalin-fixed, paraffin-embedded tumor samples using monoclonal antibodies (Thermo Scientific, USA). The percentage of stained cells and the staining intensity determined the score of positivity (1, 2 or 3) for estrogen (ER) and progesterone receptors (PR) with presence of stain in <1% cells or weak staining implying receptor negative status [17].

Restriction fragment length polymorphism (RFLP) PCR for analysis of G870A polymorphism of CCND1 on peripheral blood of all cases

Blood specimens from all participants were collected into tubes containing EDTA. DNA isolation from anticoagulated whole blood of controls as well as of breast cancer patients and children with ALL was carried out according to Sambrook et al. method [18]. Typically, 1% agarose gel was used for genomic DNA analysis, while 2% gel was used to analyze PCR products and 3% to analyze digested PCR products.

Amplification of CCND1 gene by RCR

PCR technique was used to detect CCND1 gene on chromosome 11q13 using reverse primer (CCND1R-21) with the sequence: TTTCCGTGGCACTAGGTGTC and forward primer (CCND1F' 22) with the sequence: AGTTCATTTCCAATCCGCC and the expected PCR product of 212 base pair (bp) length. Primers were HPLC purified and obtained from Fermentas Chemical Co, USA.

PCR was carried as follows: 15 µl PCR master mix [Fermentas PCR Master Mix (2X)], 3µl DNA template (0.5 µg final), forward and reverse primers 1µl

each (30 pmol) and 10 µl nuclease free water with final volume of 30 µl. PCR condition consisted of an initial denaturation of 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec, with a final extension of 72 °C for 10 min. The PCR product of 212 bp was detected by agarose gel electrophoresis using 2% agar and base pair marker of 100 bp.

Restriction fragment length polymorphism for the PCR product for detection of CCND1 G870A polymorphism

The resulting 212 base pair (bp) PCR product was digested with the restriction enzyme *Moraxella* species (*MspI*) (Fermentas) as follows:

15 µl of the resulting PCR product was digested with 2 µl of *MspI* enzyme (fast digest) and 3 µl enzyme buffer and then completed with nuclease-free water to a final volume of 30 µl, and incubated at 37°C for 30 min.

Digested PCR products were electrophoresed using 3% agarose gel to distinguish between the 175 bp band produced by the digestion of the A allele and the 141 bp band produced by the digestion of the G allele. Heterozygous state yielded both 141 bp and 175 bp bands as previously described by Le Marchand et al. [19].

Statistics

Data were analyzed using SPSS software package version 18.0 (SPSS, Chicago, IL, USA). Quantitative data were expressed using range, mean, standard deviation and median, while qualitative data were expressed as frequencies and percents. Qualitative data were analyzed using chi-square test; also exact tests such Fisher exact test and Monte Carlo test were applied to compare different groups. P value was assumed to be significant at <0.05.

Results

Acute lymphoblastic leukemia patients and control group

The age of the studied patients ranged between 4 to 12 years (mean 7.68 ± 2.51). Among the 25 patients 19 (76%) were males and 6 (24%) females. The age of the control group ranged from 3 to 12 years (mean 6.80 ± 2.88). Among the 15 healthy children 9 (60%) were males and 6 (40%) females.

All patients presented with anemia, 22 (88%) with bleeding, infections were present in 24 patients (96%),

splenomegaly and lymphadenopathy were present in all patients, hepatomegaly was present in 20 patients (80%), neurological manifestations were present in 4 patients (16%), while only 2 patients (8%) presented with testicular infiltration.

Table 1 shows the peripheral blood count and bone marrow blast cells percents of the studied ALL cases.

Immunophenotyping showed only 3 patients (12%) with T-ALL, while 22 patients (88%) were identified as B-ALL. Among the B-ALL cases, 4 (16%) were identified as Pro-B, 9 (36%) as Pre-B and 9 (36%) as common B-ALL.

All patients responded to therapy with 19 of them (76%) achieving complete and 6 (24%) partial response.

CCND1 polymorphic variants among the cases were distributed as follows: 9 (36%) were homozygous to the A allele (AA), 12 (48%) were heterozygous to the A allele, while only 4 (16%) were homozygous to G allele (GG).

In the control group only 1(6%) patient was AA, 7 (46.7%) were AG and 7 (46.7%) were GG.

Distribution of CCND1 polymorphic variants (AA, AG, GG) among patients and controls showed statistically significant difference ($p=0.040$) with significantly increased frequency of AA genotype in cases ($p=0.038$) and significantly increased frequency of GG genotype in controls ($p=0.035$; Table 2).

Figure 1 shows the PCR product of CCND1 of 212 bp as detected by agarose gel electrophoresis using 2% agarose and base pair marker of 100 bp, whereas Figures 2 and 3 show RFLP digested PCR products for analysis of G870A polymorphism of CCND1 using 3% agarose gel electrophoresis for ALL and cancer breast patients, respectively.

There was a positive risk of developing ALL with the AA genotype and the results were highly significant for AA genotype compared to GG genotype ($p=0.024$). However, no significant risk was found for AA genotype when compared to non homozygosity for AA genotype (AG+GG; $p=0.060$; Table 3).

Distribution of the different polymorphic variants among immunophenotypic categories of ALL patients T, B (Pro B, Pre B, common B) showed no statistically significant association between ALL immunophenotypes and the polymorphic variants

Table 1. Peripheral blood counts and bone marrow blast cells percentage of the studied acute lymphoblastic leukemia cases

Peripheral blood counts and BM blasts (normal range)	Mean \pm SD	Range
Hemoglobin (11-16g/dl)	6.47 \pm 1.50	4.42 – 10.57
Red blood cells (4-5.5x 10 ⁶)	2.38 \pm 0.53	1.58 – 3.41
Platelets (150-450x 10 ³)	32.68 \pm 14.07	15.0 – 63.0
White blood cells (4-13x10 ³)	8.84 \pm 7.71	1.90 – 33.40
BM blasts (0-2%)	64.28 \pm 19.09	29.0 – 90.0

BM: bone marrow, SD: standard deviation

Table 2. Comparison of Cyclin D1 polymorphic variants in acute lymphoblastic leukemia in cases and controls

Polymorphic variants	Cases (N = 25)		Controls (N = 15)		χ^2 , p-value
	N	%	N	%	
AA	9	36.0	1	6.7	0.038
AG	12	48.0	7	46.7	0.935
GG	4	16.0	7	46.7	0.035

For abbreviations see text

Table 3. Assessment of the risk of having the A allele in developing acute lymphoblastic leukemia

Polymorphic variants	Cases (N = 25)		Controls (N = 15)		OR (95% CI)	p-value*
	N	%	N	%		
AA	9	36.0	1	6.7	15.750 (1.424 – 174.246)	0.024
AG	12	48.0	7	46.7	3.0 (0.642 – 14.023)	0.257
AA + AG	21	84.0	8	53.3	4.594 (1.052 – 20.057)	0.065
GG	4	16.0	7	46.7		
AA	9	36.0	1	6.7	7.875 (0.884 – 70.151)	0.060
AG + GG	16	64.0	14	93.3		

*Fisher's exact test, OR : odds ratio. For other abbreviations see text

Table 4. Comparison of Cyclin D1 polymorphic variants between the immunophenotypic categories in acute lymphoblastic leukemia cases

	Polymorphic variant						p-value*
	AA (N = 9)		AG (N = 12)		GG (N = 4)		
	N	%	N	%	N	%	
Immunophenotyping							
T (N= 3)	1	33.3	1	33.3	1	33.3	0.736
B (N = 22)	8	36.4	11	50.0	3	13.6	
p-value**	1.000		1.000		0.422		
B	8	36.4	11	50.0	3	13.6	
Pro B (N = 4)	1	25.0	3	75.0	0	0.0	0.587
Pre B (N = 9)	5	55.6	3	33.3	1	11.1	
Common B (N = 9)	2	22.2	5	55.6	2	22.2	

* Monte Carlo test, ** Fisher's exact test

Table 5. Comparison of type of response to induction chemotherapy according to Cyclin D1 polymorphic variant in acute lymphoblastic leukemia cases

Response to chemotherapy	Polymorphic variant						p-value*
	AA (N = 9)		AG (N = 12)		GG (N = 4)		
	N	%	N	%	N	%	
CR (N = 19)	5	26.3	10	52.6	4	21.1	0.250
PR (N = 6)	4	66.7	2	33.3	0	0.0	
p-value**	0.142		0.645		0.540		

*Monte Carlo test, **Fisher's exact test CR: complete remission, PR : partial remission

Table 6. Clinicobiological parameters of breast cancer patients

Parameters	N	%
Stage		
II	15	50.0
III, IV	15	50.0
Metastasis		
Metastatic	2	6.7
Non metastatic	28	93.3
Pathology		
IDC	27	90.0
ILC	3	10.0
Menstrual status		
Postmenopausal	13	43.3
Premenopausal	17	56.7

IDC: invasive ductal carcinoma, ILC: invasive lobular carcinoma

(p=0.736 and p=0.587; Table 4).

Distribution of CCND1 polymorphic variants among ALL cases according to type of response to induction chemotherapy showed no statistically significant association between kind of response to chemotherapy and different CCND1 polymorphic variants (p=0.250; Table 5).

Furthermore, there was no statistically significant association of A allele either in heterozygous (AG) or homozygous state (AA) with the kind of response to chemotherapy in ALL cases (p=0.540).

There was no positive risk association of being homozygous to the A allele and the response to induction chemotherapy in ALL cases (p=0.142).

Breast cancer patients and control group

The age of the studied patients ranged between 29 to 76 years (mean 52.60 ± 11.17).

Table 7. Estrogen and progesterone receptor status of breast cancer patients

	N	%
ER		
-ve	2	6.7
+ve	28	93.3
+	2	7.1
++	12	42.9
+++	14	50.0
PR		
-ve	2	6.7
+ve	28	93.3
+	2	7.1
++	22	78.6
+++	4	14.3
ER / PR		
-ve	2	6.7
+ve	28	93.3
+ / +	2	7.1
++ / ++	12	42.9
+++ / ++	10	35.7
+++ / +++	4	14.3

ER: estrogen receptor, PR: progesterone receptor

The control group was age-matched with the cases, their age ranging from 26 to 75 years (mean 48.16 ± 12.82).

As shown in Table 6, 15 (50%) of the patients were identified as stage II while the rest were either stage III or IV. Only 2 (6.7%) had metastatic disease. Biopsy showed that almost 90% (n=27) of the patients had infiltrating ductal carcinoma (IDC) and the rest infiltrating lobular carcinoma (ILC). Seventeen (56%) were premenopausal and 13 (43.3%) postmenopausal.

Only 2 (6.7%) patients were ER/PR negative, while among the 28 ER/PR positive patients the degree of positivity differed (Table 7).

Table 8. Comparison of Cyclin D1 polymorphic variants between breast cancer patients and the control group

Polymorphic variants	Cases (N = 30)		Controls (N = 25)		p-value*
	N	%	N	%	
AA	8	26.7	4	16.0	1.000
AG	18	60.0	11	44.0	0.237
GG	4	13.3	10	40.0	0.032
p-value**	0.111				

*Fisher's exact test, **Monte Carlo test

Table 9. Comparison of Cyclin D1 polymorphic variants (presence or absence of A allele) between breast cancer patients and the control group

Polymorphic variants	Cases (N = 30)		Controls (N = 25)		χ^2 , p-value
	N	%	N	%	
AA + AG	26	86.7	15	60.0	0.024
GG	4	13.3	10	40.0	

Table 10. Assessment of the risk of having A allele on developing breast cancer

Polymorphic variants	Cases (N = 30)		Controls (N = 25)		OR (95% CI)	p-value*
	N	%	N	%		
AA	8	26.7	4	16.0	5.0 (0.942 – 26.530)	0.113
AG	18	60.0	11	44.0	4.091 (1.028 – 16.277)	0.055
AA + AG	26	86.7	15	60.0	4.333 (1.155 – 16.258)	0.032
GG	4	13.3	10	40.0		

*Fisher's exact test, OR: odds ratio

Distribution of different CCND1 polymorphic variants among breast cancer patients and controls showed no statistically significant difference, however the frequency of having the GG genotype was higher in the controls than in the cases when compared to the distribution of the other genotypes (p= 0.032; Table 8). Furthermore, there was statistically significant association of A allele either in heterozygous (AG) or homozygous state (AA) in breast cancer patients and its absence (GG) in the control group (p=0.024) as shown in Table 9.

Positive risk of developing breast cancer when having A allele (AA+AG) was registered compared

to GG genotype (p=0.032; Table 10). However, when homozygous A allele (AA) was compared to the non-homozygous A allele (AG+GG) no statistically significant association was found (p=0.514).

No statistically significant association between the ER/PR status and the CCND1 polymorphic variants was detected (p=1).

Distribution of different CCND1 polymorphic variants among breast cancer cases with different ER/PR status showed no statistically significant association of A allele either in heterozygous (AG) or homozygous state (AA) or its absence (GG) (p=1).

Comparison of the different CCND1 polymor-

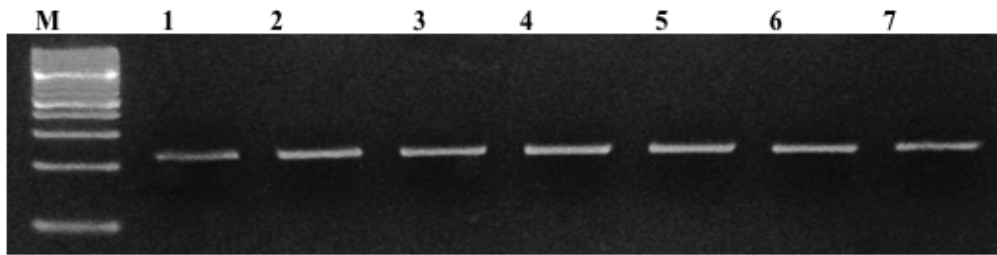


Figure 1. Agarose gel (2%) electrophoresis for PCR products. Lanes 1-7 represent PCR products for ALL patients (212 bp). Lane M represents 100bp ladder base pair marker.

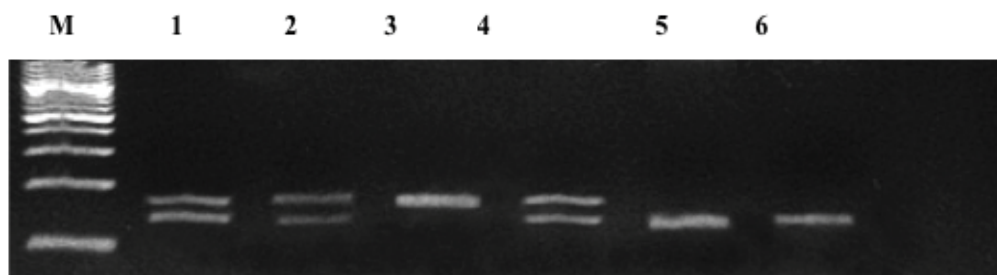


Figure 2. Agarose gel (3%) electrophoresis for RFLP digested PCR products. Lane 3 represents restricted PCR product of an ALL patient with AA genotype (175 bp). Lanes 1,2,4 represent restricted fragments of PCR products of ALL patients with AG genotype (175,141 bp). Lanes 5, 6 represent restricted PCR products of ALL patients with GG genotype (141bp).

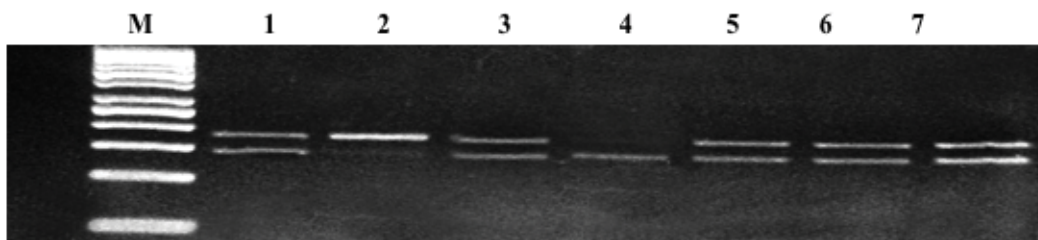


Figure 3. Agarose gel (3%) electrophoresis for RFLP digested PCR products. Lane 2 represents restricted PCR product of a breast cancer patient with AA genotype (175 bp). Lanes 1,3,5,6,7 represent restricted fragments of PCR products of a breast cancer patient with AG genotype (175,141 bp). Lane 4 represents restricted PCR products of a breast cancer patient with GG genotype (141 bp). Lane M represents 50bp ladder base pair marker.

phic variants and breast cancer patients' menstrual status at diagnosis showed no statistically significant association ($p=0.096$). Furthermore, there was no statistically significant association of A allele either in heterozygous (AG) or homozygous state (AA) or its absence (GG) with menstrual status at diagnosis ($p=0.290$).

Also, no statistically significant association was shown between the stage of disease and the CCND1 polymorphic variants ($p=0.095$). Furthermore, there was no statistically significant association of A allele either in heterozygous (AG) or homozygous state (AA) or its absence (GG) with different breast cancer stages at diagnosis ($p=0.598$).

Discussion

Cancer, in common with many other diseases, has both genetic and environmental components to its etiology. It is caused by both intrinsic factors (such as inherited mutations, hormones, and immune conditions) and environmental/acquired factors (such as tobacco, diet, radiation, and infectious organisms) [1].

The relevance of cell cycle deregulation in human cancer is being widely investigated and it affects mainly cyclin dependent kinase (CDK)/cyclin functions. Deregulation of these proteins mediates the three basic cell cycle defects, namely unscheduled proliferation, genomic instability (GIN) and chromosomal instability (CIN) [20-22].

In cancer cells, constitutive nuclear accumulation of active CCND1/CDK4 complexes can be achieved through one of several mechanisms, one of which is alternative splicing of the CCND1 transcript that resulted from a common G870A polymorphism of CCND1 that occurs in a splice donor site where the A allele was hypothesized to reduce the efficacy of the splice donor site and favor production of the alternate transcript encoding CCND1b which lacks PEST motif containing exon 5. PEST motif is critical for the degradation of CCND1; thus, transcript b (A allele) has shown to have a longer half-life than the transcript a (G allele) encoded protein [12].

Furthermore, the present study revealed a highly significant association between A allele either in heterozygous (AG) or homozygous state (AA) and cases. In addition, there was a positive risk of developing ALL with AA genotype and the results were highly statistically significant for AA genotype compared to GG genotype. However, no significant risk was found for AA genotype when compared to non homozygosity for AA genotype (AG+GG).

These observations were in concordance with those of Hou et al.[23],who studied 183 ALL cases and 190 both age- and sex-matched healthy controls, and found that CCND1 A allele was more frequent in the ALL group than in the control group.

To the best of our knowledge no other reports are published to date concerning the CCND1 polymorphic variants and ALL. However, many published reports studied the questionable association between

CCND1 polymorphic variants and the risk of developing cancer in various body organs. One of these reports is the study of Le Marchand et al. [19], who conducted a population-based case-control study in the multiethnic population of Hawaii and found that CCND1 A allele may be associated with colorectal cancer, and particularly with forms of the disease that result in severe morbidity and mortality. Moreover, Marsit et al. found that the variant AA CCND1 genotype was associated with increased relative risk of head and neck squamous cell carcinoma [24].

In the present series, there was a positive risk of developing ALL when having the AA genotype and the results were highly significant for AA genotype compared to GG genotype ($p=0.024$). However, no significant risk was found for AA genotype when compared to non homozygosity for AA genotype (AG+GG). These findings were also confirmed by the study of Hou et al. [23], where AA genotype constituted significantly higher risk of ALL when compared to GG or AG+GG genotypes.

In the present work, the distribution of the different CCND1 polymorphic variants among the immunophenotypic categories of ALL patients (T, B [Pro B, Pre B, and Common B]) showed no statistically significant association with the polymorphic variants. Furthermore, the association between the CCND1 polymorphic variants in the form of presence of A allele (AA and AG genotypes) vs. its absence (GG genotype) and the immunophenotypic results of ALL cases was investigated and showed no significant association. However, in the study of Hou et al. [23], patients with T-ALL tended to present AA more frequently than B-ALL patients, although the difference was only slightly significant ($p=0.047$). This could be attributed to the fact that the number of subjects involved in the present study was relatively small.

In the current study, the distribution of the different CCND1 polymorphic variants among ALL cases according to the kind of response to induction chemotherapy showed no significant association. Furthermore, there was no significant association of the A allele either in heterozygous (AG) or homozygous (AA) state with the kind of response to induction chemotherapy in ALL cases. However, patients who

partially responded to chemotherapy were all carrying A allele either (AA=66.7% and AG=33.3%) while all patients carrying GG genotype achieved complete disease remission. This is in agreement with the study of Hou et al. [23] in which the genotype distribution in ALL patients was significantly different between completely remitted (CR) and non completely remitted (NCR) patients. NCR patients tended to present the AA genotype more frequently than CR patients.

In the current study, TNM stage II had 50% of breast cancer patients, stage III had 43.3% and 6.6% had stage IV disease. Among the 30 patients 93.3% had non metastatic disease and almost 90% were identified as IDC while the rest had ILC.

Multiple large scale studies evaluated the CCND1 polymorphic variants and breast cancer patients from different ethnic groups; different ethnicity descents were categorized as Asian and Caucasian. The risk of breast cancer associated with the CCND1 polymorphic variants was estimated for each study by odds ratio (OR) together with 95% confidence intervals (95% CI).

In the study of Shu et al. [25], 82.3% of patients had TNM stages from 0 to II and in the study of Ceschi et al. [26] almost 70% of the cases presented at stage II or less. This reflects that high education, and health awareness, including mammographic screening and laboratory investigations, contribute to early detection of breast cancer.

In the present series, the frequency of having the GG genotype was higher in the control group than in cases when compared to the distribution of the other genotypes. Furthermore, there was a statistically significant association between the A allele either in heterozygous (AG) or homozygous state (AA) and breast cancer patients.

In agreement with the present study, Yu et al. [27] found that the frequency of GG genotype was higher in the control group, while the frequency of A allele either in homozygous (AA) or heterozygous (AG) state was higher in cases. On the other hand, Krippel et al. [28] reported that CCND1 genotype frequencies were similar among patients and controls.

Abramson et al. [29] analyzed CCND1a (which

is postulated to be correlated to the G allele) and CCND1b (which is postulated to be correlated to the A allele expression [21] in a cohort of women with early-stage breast carcinoma. They noticed that AA genotype was found in 22% of the cases, in 4% of the AG genotype and in 22% of the GG genotype of the cases.

In the current study, there was a positive risk of developing breast cancer for those having the A allele either in homozygous (AA) or heterozygous (AG) state when compared to GG genotype. However, homozygosity for the A allele (AA) when compared to GG genotype and when compared to non homozygosity for A allele (AG+GG) showed no significant risk association.

In concordance with our findings, Shu et al. [25] showed that the A allele, either in homozygous (AA) or heterozygous (AG) state when compared to GG genotype was only weakly associated (borderline significance) with the risk of breast cancer, while the AA genotype when compared to GG genotype was not related with positive risk of developing breast cancer. This shows a possible oncogenic effect for the A allele which is maintained in both homozygous and heterozygous states.

This is also in agreement with the results of Yu et al. [27] who showed that there was a positive risk of developing breast cancer when having the A allele either in homozygous (AA) or heterozygous (AG) state when compared to GG genotype. Moreover, no positive risk association was found when AA genotype was compared to non homozygosity for A allele (AG+GG).

Krippel et al. [28], showed that no risk of developing breast cancer for those having the A allele either in homozygous (AA) or heterozygous (AG) state, while there was a borderline risk association between AA genotype and breast cancer (OR =1.25).

In the study of Onay et al. [30], homozygosity for the A allele (AA) when compared to GG genotype showed a positive risk of developing breast cancer in both the Ontario and the Finland samples. However, the presence of the A allele (AA+AG) when compared to GG genotype showed no positive risk association in both Ontario and Finland populations.

Positive risk of developing breast cancer when having the A allele either in homozygous (AA) or heterozygous (AG) state is a common finding among the

discussed studies which included large-scale studies, like Shu et al. and Yu et al. studies and small-scale studies like ours. Nevertheless, the power of homozygosity for the A allele in pertaining a positive risk of breast cancer is a point of discordance between studies in which the study of Onay et al. [30] agreed with this finding, while the studies of Yu et al. [27] and Shu et al. [25] and our study didn't show significant association between homozygosity for the A allele and the risk of breast cancer development.

In the present study, comparing between the different CCND1 polymorphic variants and the Estrogen ER/PR status in breast cancer patients showed that 80% of the cases having the A allele were ER/PR positive. This is in concordance with Abramson et al. [29] study in which 74% of breast cancer cases carrying the A allele were ER/PR positive. On the other hand, the study of Shu et al. [25] reported that only 30% of the cases carrying the A allele were ER/PR positive. Obviously, the rationalization of these discrepant results represents a real difficulty. However, further extrapolation of the confounding factors governing the expression of these hormonal receptors might explain this discordance.

In the present study, comparison between the different CCND1 polymorphic variants and breast cancer patients' menstrual status at diagnosis showed that about 94% of the premenopausal patients and 76% of the postmenopausal patients had the A allele ($p > 0.05$). Similar results were described by Shu et al. [25] and Yu et al. [27].

However, in the study of Abramson et al. [29] 39% of the premenopausal breast cancer patients were carrying the A allele. As discussed above, these results drew attention towards considering whether other confounding pathobiologic factors might stand behind these unexplained discrepancies.

In the current work, distribution of the different CCND1 polymorphic variants among different stages of breast cancer patients showed that almost 93.3 % of patients with TNM stage III and IV carried the A allele, while 80% with TNM stage II carried this allele ($p > 0.05$).

Similar results were described by Shu et al. and Ceschi et al. In the study of Shu et al. [25], 76.6% of women with TNM stages III and IV carried the A al-

lele, while 81.9% with TNM stages 0- II carried the A allele. In the study of Ceschi et al. [26], 76.5% of patients with TNM stages $> II$ (advanced stage disease) were carrying the A allele, while 79% with TNM stages 0-I carried the A allele.

In recent years, accumulating evidence from large numbers of studies has implicated that the CCND1 G/A870 polymorphism is a modulator of cancer risk and prognosis. Moreover, these studies presented evidence of associated risk of the A allele with the development of different types of cancer.

The present study supports these findings in that homozygosity for CCND1 A allele was associated with ALL patients and was a risk factor for ALL development, while the presence of the A allele, whether homozygous or heterozygous, was associated with breast cancer cases and was a risk factor for breast cancer development. Homozygosity for CCND1 G allele was associated with the control group.

We recommend expansion of further studies to include more patients and healthy controls in order to acquire firm data concerning the CCND1 polymorphic variants and evaluate patients' disease free survival and overall survival in relation to the CCND1 polymorphic variants. In addition, we invite future research efforts to validate the association of CCND1 polymorphic variants with human malignancies.

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Non-Hodgkin lymphomas and carrier state of viral hepatitis B and C

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Summary

Purpose: To establish the characteristics and prognosis of newly diagnosed patients with non-Hodgkin lymphoma (NHL), who were carriers of hepatitis B (HBV) and C (HCV) viral infection.

Methods: 542 patients with NHL, diagnosed and treated in the University Hospital "Sv. Georgi", Plovdiv, were retrospectively analysed. Two NHL patient groups were created – the study group, consisting of 33 patients with NHL positive for HBV and HCV, and the control group, consisting of 40 randomly assigned patients with NHL and negative serology for hepatitis. Study and control groups were compared for basic characteristics and survival.

Results: The prevalence of hepatitis B surface antigen (HBsAg) among newly diagnosed patients was 5.72% and of HCV 1.84 %. Association with hepatitis viruses was more frequent in indolent than in aggressive NHLs ($p=0.044$). Liver dysfunction was registered more often in the study group ($p=0.002$). Reactivation of HBV infection was registered in 5 patients (12.19%) from the study group. There was no statistically significant difference between survival rate of patients in the study group and in the control group ($p=0.738$).

Conclusion: Hepatitis virus carrier state did not alter significantly the clinical course and disease prognosis (remission rates and survival) in our patient group. We recommend the routine testing for hepatitis infection in patients newly diagnosed with NHL in order to collect more data needed for the establishment of a possible causal relationship between hepatitis viruses and NHL. Since antiviral prophylaxis could positively impact the course of lymphoma treatment, national guidelines for the management of patients with hepatitis infection and NHL will prove to be necessary for the clinical practice.

Key words: hepatitis B, hepatitis C, non-Hodgkin lymphomas

Introduction

It is considered that one quarter of malignant disorders have infectious etiology. The etiopathogenetic role of viruses has been recognized for the development in some carcinomas and sarcomas [1], e.g. HCV and hepatocellular carcinoma, EBV (Epstein-Barr virus) and nasopharyngeal carcinoma, HIV (human immunodeficiency virus) and Kaposi sarcoma, HPV (human papilloma virus) and uterine cervix carcinoma, and human herpes virus-8 and Kaposi sarcoma. There is fewer data on the role of viruses in the lymphomagenesis: EBV is isolated in 100% of the patients with Burkitt lymphoma; HTLV-1 (human T cell leukemia type I) and peripheral T-cell lymphoma; and HCV and mixed cryoglobulinemia (preneoplastic lymphoproliferative disorder).

The causal relationship between HBV and HCV infection and NHL lymphomas has been a subject of intense research for the past 20 years [2]. Most of the studies published have been conducted in regions with high incidence HCV infection and point to certain etiopathogenetic relationship between both diseases. However, the causality of lymphoproliferative disorders related to HCV and HBV carrier state remains uncertain. The predictive value of the virus carrier state related to the treatment outcomes (in terms of viral reactivation) as well as the necessity of antiviral prophylaxis haven't been established yet.

The purpose of this study was to analyse the characteristics and survival of patients newly diagnosed with NHL, who were carriers of HBV and HCV.

Methods

Patients

This retrospective study included 542 patients with newly diagnosed NHL, treated at the University Hospital "Sv. Georgi" – Plovdiv and the Comprehensive Oncology Centre – Plovdiv from 2008 to 2011. Positive hepatitis virus carrier state was registered in 41 NHL patients. For some of the patients no complete data were available for statistics.

We analysed the characteristics of two NHL patient groups – the study group, with 33 NHL patients positive for HBV and HCV, and the control group, with 40 randomly assigned patients with NHL and negative serol-

ogy for hepatitis. Both groups were compared by main clinical parameters and survival. Clinical stage was defined according Ann-Arbor staging system. The therapeutic response was evaluated according to the International Working Group Response Criteria for NHLs.

Follow up

Patients in remission were followed at 3- to 6-month intervals in the outpatient department until April 2012 (end of the study). The median follow-up time was 18 months (range 1-143).

Statistics

Chi-square test and Student's t-test were used for comparison of both groups. Survival analysis was performed by the Kaplan-Meier method [3]. Comparison of survival curves was based on the log rank test. Survival time was measured from the date of diagnosis and endpoints were taken as death from all causes. Statistical analysis was carried out using the SPSS v.17.0 statistical package.

Results

The study group included 31 HBV (+) NHL and 10 HCV (+) NHL patients. Positive carrier state among newly diagnosed NHL patients was 5.72% for HBV and 1.84% for HCV.

Comparison of both groups

No statistically significant difference was found when the study and the control group were compared by demographic characteristics: age (t-test=1.28, p=0.202) and sex ($\chi^2=2.37$, p=0.124), frequency of extranodal localization ($\chi^2=0.025$, p=0.874), complete remission rate ($\chi^2=0.139$, p=0.709) (Table 1).

There was a trend of diagnosing patients from both groups in advanced clinical stage (Table 1), and most stage IV patients belonged to the study group (Figure 1).

Viral hepatitis carrier state was found more frequently in indolent lymphomas ($\chi^2=4.06$, p=0.044) with significant difference between the study and control group (Figure 2).

Liver dysfunction was registered significantly more often in the study group ($\chi^2=9.91$, p=0.002; Figure 3).

Table 1. Characteristics of patients in the study and control group

Clinical parameters	Study group (N=33) N %		Control group (N=40) N %		p-value
Age, years (mean±SE)	59.5±1.81		63.5±2.01		NS
Sex					
Male	13	36.1	23	63.9	NS
Female	20	54.1	17	45.9	
Clinical stage					
Localized	10	40.0	15	60.0	NS
Generalized	23	47.9	25	52.1	
Remission rates (complete and partial)	19	46.3	22	53.7	NS
Extranodal localization	15	45.5	18	54.5	NS
Histological subtype					
Indolent	22	55.0	18	45.0	0.044
Aggressive	10	31.3	22	68.7	
Liver dysfunction	11	33.3	2	5.0	0.002

NS: non significant

Survival

The mean survival time of patients in the study group was 54.6 months (95% CI 29.5-79.8). The survival of patients in the control group (42.6 months, 95% CI 24.1-61.1) was longer than that of study group, but without statistically significant difference (log rank=0.112, p=0.738; Figure 4).

Reactivation of viral hepatitis infection

Reactivation of HBV infection due to chemotherapy was registered in 5 patients (12.19%) from all hepatitis virus positive patients. In one patient reactivated viral infection in conjunction with chemotherapy ended in fatal outcome attributable to severe liver damage from the virus.

There were no cases of acute viral hepatitis infection in the control group.

Discussion

In the past 20 years a possible causal relationship between the viruses of infectious hepatitis and NHL has been proposed in the literature, most of the studies pointing to a relationship between HCV and NHL [2,4]. A meta-analysis of 15 case-control studies and 3 prospective studies (9 of which had not been included in previous meta-analyses) found an increased relative risk of developing NHL among HCV-

positive subjects, irrespective of histological subtype [5]. Evidence for causality is the successful achievement of complete remission in some cases of indolent lymphoma solely with the administration of antiviral therapy [6]. Data on the role of HBV in lymphomagenesis is scarce and controversial. A 14-year follow-up of HBV (+) subjects also determined an increased risk of NHL, but only in certain subtypes [7].

The reported frequency of HBV and HCV carrier state among newly diagnosed patients with NHL in our study is consistent with the current literature data: 1.89-2.8% prevalence of HCV and 3.7-23.5% of HBV [8-10]. Frequencies vary, depending on the infectious rates of the population in a certain region. Our data corresponds with published data for the Black Sea region [11]. On the other hand, the reported frequency of HBV and HCV among patients with NHL in our study was higher than the rate of healthy population in the Plovdiv region (3.8% for HBV and 1.3% for HCV), which could be suggestive of a possible causal relationship [12]. Published data suggests a possible etiopathogenetic relation between hepatitis viruses and certain NHL subtypes [13]. Some authors indicate higher frequency of the association of aggressive NHL subtypes with viral hepatitis carrier state [4,8]. Others find higher incidence of indolent lymphomas (M. Waldenström) among HCV carriers [9]. Suppos-

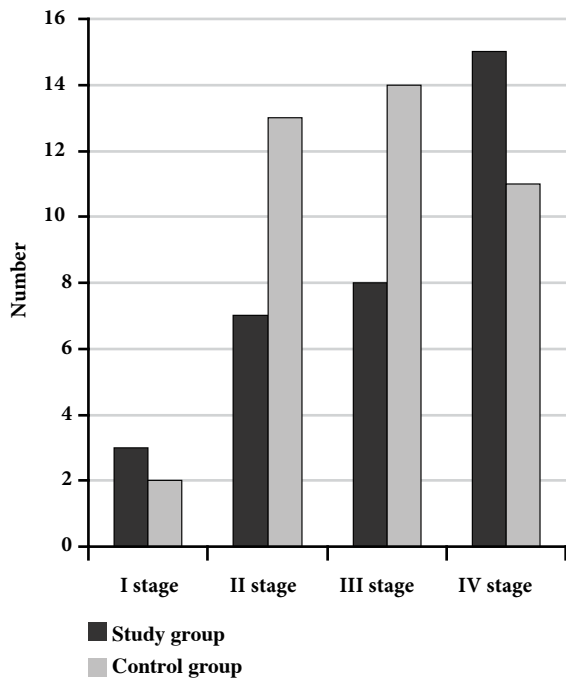


Figure 1. Patient distribution according to clinical stage in the study and control groups ($p>0.05$)

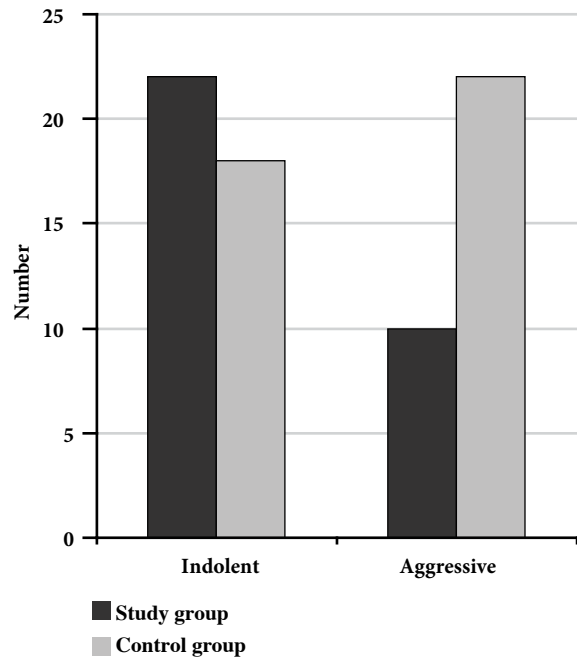


Figure 2. Patient distribution according to histological type ($p=0.044$).

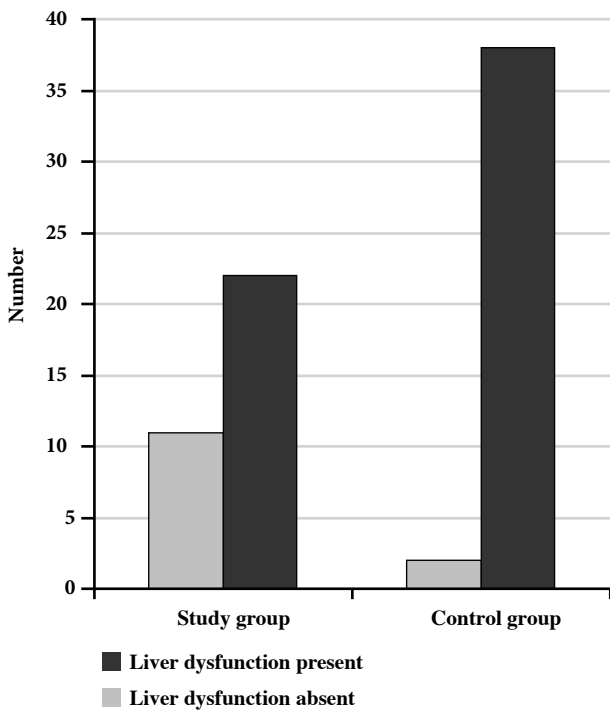


Figure 3. Patient distribution according to the presence of liver dysfunction ($p=0.002$).

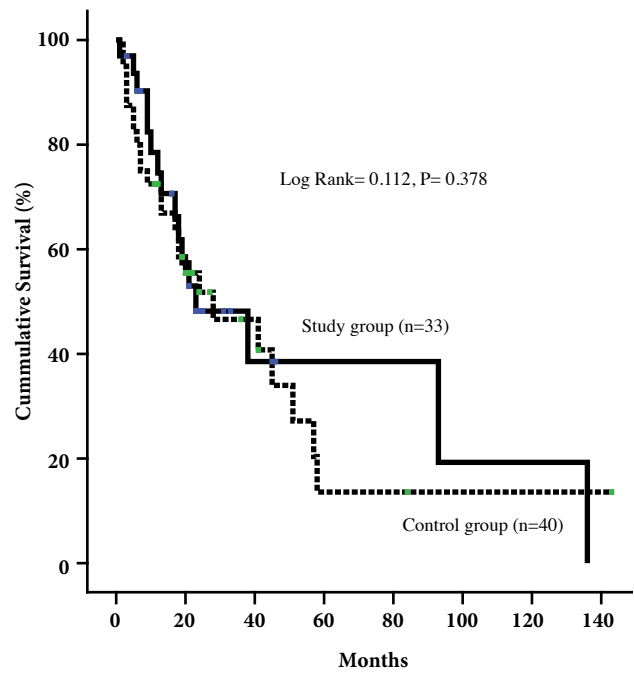


Figure 4. Survival of NHL patients from the study and control groups.

edly, higher prevalence of HCV is found in aggressive diffuse large B-cell lymphomas (DLBCL) after transformation from indolent NHL. In our study we found more frequent association of hepatitis viruses with indolent lymphomas, which could be supportive of the latter statement.

Viral infections confer certain risk for developing NHL, mostly due to causing primary or secondary immune deficiency. Unlike known oncogenic viruses and while it is typical for HBV DNA, hepatitis C RNA does not integrate within the host's genome. Possible mechanisms of HCV involvement in lymphomagenesis are currently being discussed in the literature. A direct role of HCV and its presence in hematopoietic cells is questionable. Most authors support the concept of chronic B-cell stimulation by viral antigens, leading to polyclonal B-cell expansion, immune dysregulation and thus to B-cell malignant lymphoproliferative disorder. Triggering mechanism is the binding of E2-protein from the viral envelope to the B-cell surface complex CD19/CD21/CD81, followed by clonal activation and chronic B-cell proliferation [2]. Data on the prognosis of patients with NHL, infected with HBV and HCV is controversial as well. High percentage of viral reactivation (60%) in HBV (+) patients due to immune suppression has been reported in the literature [15-17]. This risk persists up to 6 months after chemotherapy cessation. According to other data 22.3 % of NHL patients develop acute hepatitis while on chemotherapy with mortality rate of 3.7% [18-20]. Some major centers such as the Memorial Sloan Kettering Cancer Centre – USA have adopted routine antiviral prophylaxis against HBV infection in NHL patients at least 6 months following completion of chemotherapy. Most frequently used antiviral agents are Lamivudine, Entecavir, Tenofovir and others.

Our patients did not receive antiviral prophylaxis. We registered a rather low frequency of viral reactivation during chemotherapy (12.19%) and one lethal case associated with viral reactivation and fulminant liver damage. This prompts the expectation of a positive impact of antiviral prophylaxis during the course of lymphoma treatment and urges the necessity of pharmacoeconomic analysis.

Conclusion

In this study we determined an infectious rate of 5.72% for HBV and 1.84% for HCV among patients newly diagnosed with NHL. Hepatitis virus carrier state seems not to affect significantly the clinical course and prognosis of NHL. We recommend the routine testing for hepatitis infection in patients with newly diagnosed NHL in order to collect more data needed for the establishment of possible causality. Since antiviral prophylaxis could positively impact the course of lymphoma treatment, national guidelines for the management of patients with hepatitis infection and NHL will prove to be necessary for the clinical practice.

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The role of VEGF and other parameters in tracking the clinical course in metronomic chemotherapy

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Summary

Purpose: The purpose of this study was to investigate the effect of metronomic chemotherapy on serum vascular endothelial growth factor (VEGF) levels in cancer patients.

Methods: The study included 11 metastatic cancer patients who received daily 50 mg cyclophosphamide and biweekly 5 mg methotrexate *per os* as metronomic chemotherapy. Bevacizumab together with FOLFIRI chemotherapy was administered as anti-angiogenic treatment in another group of 16 metastatic colorectal carcinoma patients. Furthermore, VEGF levels of 10 healthy individuals and 5 cord blood samples served for comparisons. VEGF levels of patients before therapy and 3 months after treatment were analyzed and compared.

Results: Serum VEGF levels prior to metronomic chemotherapy were higher compared with the healthy controls ($p=0.0001$). Similarly, serum VEGF levels prior to the bevacizumab-based chemoimmunotherapy were significantly higher compared with the healthy controls ($p=0.005$). In patients on metronomic chemotherapy VEGF levels showed non significant decrease ($p=0.075$). On the contrary, VEGF levels decreased significantly ($p=0.002$) with bevacizumab treatment.

Conclusion: Serum VEGF levels may be used for assessing of the efficacy of anti-angiogenic therapies.

Key words: bevacizumab, chemotherapy, metronomic chemotherapy, VEGF

Introduction

Currently the first and second leading causes of human deaths worldwide are characterized by lack of angiogenesis (cardiovascular diseases) and excess of angiogenesis (cancers). Conventional response parameters are not sufficient enough to assess the efficacy of the therapies targeting angiogenesis. Furthermore, the use of conventional cytotoxic drugs is restricted by their maximal tolerable dose (MTD). Although anticancer drug therapies result in the cure of a limited, and disease control in a substantial number of patients, chemotherapy given in MTD causes noticeably long and short term complications. Furthermore, with their notoriously dynamic and heterogeneous genetic instability, cancer cells have a tendency to develop resistance to multiple chemotherapeutic drugs [1]. An alternative method of administration with increased anticancer effects through continuous administration of cytotoxic drugs in low doses to avoid the onset of dose-restricting side effects and thus to eliminate the need for resting periods, is drawing increased attention. These forms of chemotherapy are called “metronomic chemotherapies”. By means of targeting the activated endothelial cells that are proliferating much more slowly than tumor cells, metronomic therapies inhibit the growth of tumor cells resulting from insufficient neovascularization. It was not until recently when the beneficial effects of antiangiogenic treatment based on metronomic chemotherapy or other methods in cancer patients could be demonstrated [2,3].

In fact, a variety of protocols which were earlier called “sustained chemotherapy” in various cancers could well be described as metronomic chemotherapy: daily mercaptopurine + weekly methotrexate used successfully in leukemia; daily cyclophosphamide + weekly vincristine effective in neuroblastoma; the weekly use of vincristine in Wilms’ tumor and the UFT schemes in lung adenocarcinoma are some examples [4,5].

VEGF is also known as the vascular permeability factor. Melder et al. [5] have shown that VEGF promotes VCAM-1 (vascular cell adhesion molecule 1) and ICAM-1 (intercellular adhesion molecule 1) in endothelial cells. This stimulation may result in the adhesion of VLA-4 (very late antigen-4) and CD 18 on the sur-

face of the natural killer (NK) cells and the active NK cells on endothelial cells through the specific interaction between the endothelial VCAM-1 and ICAM-1. These effects may provide an explanation for the previously observed preferential adhesion of NK cells activated by IL-2 against tumor vascularization [6,7]. Gabrilovich et al. suggested that VEGF could have an inhibitory effect in the maturation of the cells that provide professional antigens such as dendritic cells [8]. These findings suggest that VEGF may facilitate tumor growth, while at the same time allowing the escape of the tumor from the stimulation of an immune response. Studies performed at various laboratories have clarified the essential role of VEGF in the arrangement of normal and abnormal angiogenesis. Particularly, the loss of even a single VEGF allele results in embryonic death and reveals the role assumed by this factor in the development and diversion of the vascular system [9,10]. The human VEGF gene is composed of 8 exons and 7 introns. Initially, the alternative portion of the exon results in the form of 4 different isoforms (VEGF121, VEGF165, VEGF189, VEGF206). These contain 121, 165, 189 and 206 amino acids respectively. VEGF165 is the predominant isoform and lacks the portion coded with exon 6. VEGF121 does not contain the portions coded by exon 6 and 7. The less frequent variants of the alternative portion have been described separately. These are VEGF145 and VEGF183 [11]. Human VEGF is a 45 kDa, heparin-binding homodimeric glycoprotein. The properties of the natural VEGF match to those of VEGF165. VEGF121 is a non-heparin binding, acidic polypeptide. VEGF189 and VEGF206 are highly basic and bind heparin with high affinity. However, while VEGF121 is an easily spreading protein, VEGF189 and VEGF206 are almost exclusively present in the extracellular matrix (ECM). VEGF165 has intermediate properties. Despite secretion, a substantial portion remains on the cell surface and in the ECM. Loss of the VEGF’s heparin binding region results in substantial loss of mitogenic activity [12]. Several studies have demonstrated that the combined use of anti-VEGF therapy with chemotherapy or radiotherapy resulted in anti-tumor effect higher than the one achieved by the individual use of both treatments.

Clinical trials addressing cancer patients are continued with various VEGF inhibitors that contain

humanized monoclonal antibodies (rhuMab VEGF), one anti-VEGFR-2 antibody, small molecules that inhibit VEGFR-2 signal transmission and one soluble VEGF receptor against VEGF. Phase II clinical data provide initial evidence for rhuMab VEGF. When combined with conventional chemotherapy, rhuMab VEGF increases survival in patients with metastatic colorectal cancer [13].

The purpose of this study was to investigate the effect of angiogenesis inhibitors on endothelial cells by measuring the serum VEGF levels of patients on metronomic chemotherapy and patients receiving FOLFIRI chemotherapy plus the anti-VEGF monoclonal antibody bevacizumab.

Methods

The study was approved by the local ethics committee, and written informed consent was obtained from all patients and healthy volunteers.

Patients and treatment protocols

The study included 11 metastatic cancer patients who received daily 50 mg cyclophosphamide and biweekly 5 mg methotrexate per os as metronomic chemotherapy. These patients had received many lines of chemotherapy in the past.

Metronomic chemotherapy was given continuously until unacceptable toxicity or disease progression. Patients were examined monthly with routine physical examination together with hemotological and biochemical tests for toxicity assessment.

Bevacizumab (5 mg/kg) along with conventional FOLFIRI chemotherapy (irinotecan 180 mg/m², 5-fluorouracil 3000 mg/m², leucovorin 200 mg/m²) were given biweekly. This anti-angiogenic therapy was used as first-line treatment to another group of 16 metastatic colorectal carcinoma patients.

In addition, VEGF levels of 10 healthy volunteers and 5 samples of cord blood were assessed. VEGF levels of patients prior to therapy and 3 months after treatment were analyzed. Then, these levels were correlated with the patient clinical outcomes.

Lab methods

Blood samples were collected prior to and 3 months

after the beginning of therapy from patients receiving metronomic chemotherapy and bevacizumab plus FOLFIRI at the Medical Oncology Clinic. The serum of the collected blood samples was separated (2000 g, 7 min) and kept at -80°C.

Blood samples were also collected from 5 healthy subjects of 25±5 years of age and 5 in the 50±5 age group. The serum of the collected blood samples was separated (2000 g, 7 min) and kept at -80°C.

Despite the finding of very low VEGF level in the cord blood, 5 different cord blood serums were also used for control purposes. The patient serum samples were assayed using ELISA (Enzyme-Linked Immunosorbent Assay) for VEGF levels at the initial stage, and 3 months after initiation of therapy. Two microwells not containing serum served as negative control. The study used Biosource brand VEGF165 ELISA kit. All serums tests were carried out in duplicate.

Statistics

Statistical analyses were performed using SPSS version 15 for Windows (SPSS Inc., Chicago, IL, USA). Comparisons between 3 groups were performed using the Kruskal-Wallis Variance analysis, while the dual group comparisons were performed by the Mann-Whitney U test with Bonferonni correction. The VEGF levels of the groups at different times were compared using the Wilcoxon test. A p value below 0.05 was considered as significant. The values in Tables and Figures were given as average, SD (standard deviation), and range.

Results

The study included 11 patients (7 females, 4 males, average age 55 years, range 42-69) receiving metronomic chemotherapy, 16 patients (4 females and 12 males, average age 55.1 years, range 28-70) receiving bevacizumab plus FOLFIRI therapy, 5 healthy individuals in the 25±5 years age group (2 females and 3 males, 27.6 years on average), and 5 healthy subjects (5 males, 51 years of age on average) in the 50±5 years age group as controls. Furthermore, 5 cord blood samples from different individuals with low VEGF levels were used for controlling purposes for the reliability of the ELISA analysis.

The age, diagnosis, pre- and post-metronomic chemotherapy patient serum VEGF levels are shown in Table 1.

The age and diagnosis of serum VEGF levels of the patients receiving bevacizumab plus FOLFIRI therapy are shown in Table 2.

The age and serum VEGF levels of the healthy

controls are shown in Table 3.

The VEGF serum levels in the 5 cord blood of individuals with low VEGF levels and who served for the reliability of the ELISA analysis were 39, 44, 43.2, 38.6, and 33.4 pg/ml respectively (average 39.64 pg/ml).

The average, SD and range of VEGF of the patients

Table 1. Age, diagnosis, pre- and post-metronomic chemotherapy serum VEGF levels of patients receiving metronomic chemotherapy

<i>Patients</i>	<i>Age (years)</i>	<i>Diagnosis</i>	<i>VEGF level before metronomic chemotherapy (pg/ml)</i>	<i>VEGF level after 3 months metronomic chemotherapy (pg/ml)</i>
GB	48	Breast Ca	450	676
FA	69	Breast Ca	194	299
HT	64	Breast Ca	280	128.5
CC	42	Breast Ca	660	410
MS	60	Breast Ca	521.5	518.5
NK	62	Ovarian Ca	600	96
HU	54	Lung Ca	202	160.4
RY	56	Breast Ca	300	350
FD	49	Breast Ca	596	240
FS	52	Lung Ca	898	197.5
HO	49	Nasopharynx Ca	1200	368

Table 2. Age, diagnosis, and serum VEGF levels of patients receiving bevacizumab plus FOLFIRI therapy

<i>Patients</i>	<i>Age (years)</i>	<i>Diagnosis</i>	<i>VEGF level before bevacizumab therapy (pg/ml)</i>	<i>VEGF level after 3 months bevacizumab therapy (pg/ml)</i>
M.AK	61	Colon Ca	206	62
AY	60	Colon Ca	207	78.04
CY	53	Colon Ca	121	119.1
NK	65	Colon Ca	81	121
HK	28	Colon Ca	46	130
MC	52	Colon Ca	597	118
HK	40	Colon Ca	602	117
MS	62	Colon Ca	129	89
HE	45	Colon Ca	446	115
AK	53	Colon Ca	450	130
BO	56	Colon Ca	187	117
HK	66	Colon Ca	216	117
UK	64	Colon Ca	325	127
SC	58	Colon Ca	295	115
MK	70	Colon Ca	178	119
M.AT	50	Colon Ca	600	116

are shown in Table 4.

The average VEGF serum level of healthy individuals was 119.5 pg/ml, SD was 35.1, and range 47-195 pg/ml.

The difference of the average VEGF levels was statistically significant when VEGF levels of the patients receiving metronomic chemotherapy, those receiving bevacizumab plus FOLFIRI and healthy subjects were compared. Kruskal–Wallis analysis showed that the VEGF levels of healthy individuals were significantly lower ($p=0.0001$) vs. those of the patients. Serum VEGF levels of both age groups in the control population of which half was approximately 25 and the other half around 50 years old were similar. On the contrary, VEGF values in the control cord blood corresponded to approximately half of the VEGF (pg/ml) value in both groups.

It is interesting that the medical history of the individual with the lowest VEGF level in the control group (his VEGF was 46.9 pg/ml vs. healthy control group average 120.7 pg/ml) revealed that both, the individual and his family were suffering of atherosclerosis. The subject with the highest level of VEGF in the control group (his VEGF was 195 pg/ml vs. healthy control group average 120.7 pg/ml) was found to be a carrier of sickle cell anemia while he had no family history of atherosclerosis. A comparison between the initial VEGF levels of patients receiving metronomic chemotherapy and bevacizumab plus FOLFIRI therapy showed that the VEGF levels of metronomic chemotherapy pa-

tients were significantly higher ($p=0.030$) according to Mann-Whitney U test analysis. Mann-Whitney U test analysis showed that the initial VEGF levels of patients receiving metronomic chemotherapy were significantly higher ($p=0.0001$) than those of healthy individuals. Also, Mann-Whitney U test analysis showed that the initial VEGF levels of patients receiving bevacizumab plus FOLFIRI were significantly higher ($p=0.005$) than those in healthy subjects. A comparison between the VEGF levels after 3 months of patients receiving metronomic chemotherapy and bevacizumab plus FOLFIRI treatment showed that the VEGF levels of the metronomic chemotherapy patients were significantly higher ($p=0.0001$) according to Mann-Whitney U test analysis. Despite the fall in the initial and 3-month average VEGF levels of patients receiving metronomic chemotherapy, this decrease was not significant according to Wilcoxon analysis ($p=0.075$; Figure 1). However, the decrease in VEGF levels 3 months later of patients receiving bevacizumab plus FOLFIRI was significant ($p=0.002$) according to Wilcoxon analysis.

Discussion

VEGF is the most important target of anti-angiogenic therapy and is secreted by approximately 60% of human tumors. Long life expectation of cancer patients bears the risk of the production of high amounts of angiogenic proteins associated with tumor cells' mutations. For example, while most breast cancers secrete only VEGF at the time of diagnosis, relapsed cases of breast cancers secrete approximately 5 different types of angiogenic proteins. Angiogenetic inhibitors prevent tumor progression by affecting the function and/or proliferation of the endothelial cells. The purpose of this study was to ensure proper observation of the results achieved through the effects of the angiogenetic inhibitor bevacizumab on endothelial cells through estimations of serum VEGF levels in patients receiving metronomic chemotherapy and bevacizumab plus FOLFIRI treatment [9-14].

Bevacizumab is an anti-VEGF monoclonal antibody that binds and neutralizes all human VEGF-A isoforms and bioactive proteolytic fragments. In two of our recent studies we observed changes in sTRAIL levels associated with outcome in response to beva-

Table 3. Age and serum VEGF levels of the healthy controls

<i>Healthy controls</i>	<i>Age (years)</i>	<i>VEGF level (pg/ml)</i>
DE	28	129
AD	30	126
KO	30	118
SU	22	119
DB	28	125
TK	49	46.9
BS	49	118.6
EY	49	195
ME	54	120
OA	54	113

cizumab therapy; serum IL8 levels were decreased in all patients, however, this change was not correlated with disease outcome [15,16].

Factors such as the lack of frequent resistance to drugs that target directly the tumor cells, tumor endothelial cell proliferation 50-100 times faster than normal endothelial cells, the presence of marks which are available on active endothelial cells but not on the inactive (non-proliferating, silent) ones, the highly rare occasions of anti-angiogenic drug-related side effects in adults due to limited angiogenesis, easy access to the endothelial cells through blood circulation, and the killing of tumor cells in large amounts through damage to several micro vessels are the advantages which are expected to bolster anti-angiogenic therapy along with conventional methods of treatment in the near future. The most important issue, however,

would be the inability to track therapeutic activity with conventional response parameters (Response Evaluation Criteria In Solid Tumors [RECIST]) since the RECIST parameters are based on the shrinkage of the tumor tissue. However, this does not occur always in line with tumor vascularization.

This study was conducted on 11 patients (7 females, 4 males, 55 years of age on average) receiving metronomic chemotherapy, and on 16 patients (4 females, 12 males, 55.1 years on average) receiving bevacizumab plus FOLFIRI treatment. Of the patients receiving metronomic chemotherapy, 6, 2, 2 and 1 had breast, ovarian, lung, and nasopharyngeal cancer, respectively. All 16 patients receiving bevacizumab therapy had colon cancer.

A comparison of the initial VEGF levels between the patients receiving metronomic chemotherapy

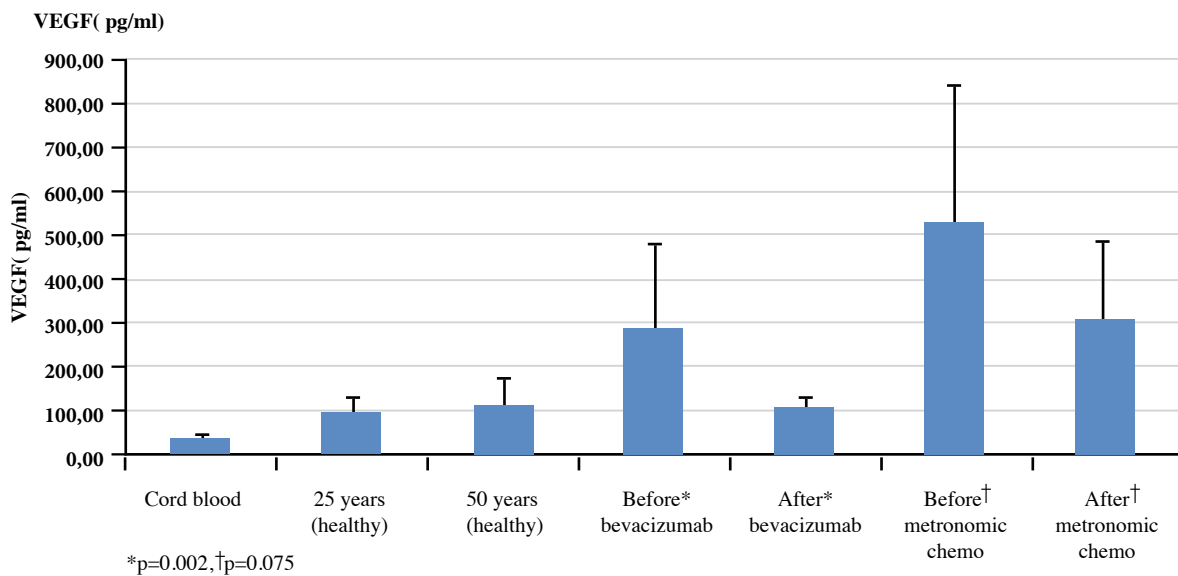


Figure 1. The average VEGF levels of healthy individuals and patients.

Table 4. Average, standard deviation (SD) and range of patient VEGF data

	VEGF levels before therapy (pg/ml)			VEGF levels after 3 months (pg/ml)			p-value (before vs after)
	Mean	SD	Range	Mean	SD	Range	
Metronomic chemotherapy (N=11)	520	308	194-1200	299	176	96-676	0.075
Bevacizumab plus FOLFIRI chemotherapy (N=16)	211	189	46-602	117	18.9	62-130	0.002

and those with bevacizumab treatment resulted in significantly higher VEGF levels ($p=0.030$) of those with metronomic chemotherapy. Patients with metronomic chemotherapy were those who received a large number of chemotherapies at the earlier and later stages. Conventional chemotherapy might stimulate angiogenesis because it is usually given every 3-4 weeks which includes a resting period of 2-3 weeks. Endothelial cells are killed during the time of chemotherapy, however they reproduce themselves during these resting periods. On the contrary, bevacizumab patients had not received prior therapy. Another reason for the difference in the initial VEGF levels in the metronomic treatment group might be the multiple organ involvement (brain, lung) and particularly bone metastases, and liver metastasis in those receiving bevacizumab treatment.

The initial VEGF levels of patients receiving metronomic chemotherapy were significantly higher ($p=0.0001$) when compared to healthy individuals. Likewise, the initial VEGF levels of patients receiving bevacizumab plus FOLFIRI therapy were also significantly higher ($p=0.005$) compared with healthy subjects.

These observations support the idea that VEGF secretion and the angiogenetic process are high in metastatic cancers. A comparison between the VEGF levels after 3 months of patients receiving metronomic chemotherapy and bevacizumab treatment showed that the VEGF levels of the metronomic chemotherapy patients were significantly higher ($p=0.0001$), while the bevacizumab therapy yielded an average decrease of 94.5 pg/ml in VEGF levels; the average decrease of VEGF levels achieved with metronomic chemotherapy was 221 pg/ml. Although there was a decreasing trend in the initial and 3-month later VEGF levels in patients subjected to metronomic chemotherapy, this decrease was not statistically significant ($p=0.075$). A study by Artac et al. performed in our country showed a relation between the decrease of serum VEGF levels and non-progression survival in 8 patients with metastatic breast cancer who received metronomic cyclophosphamide plus etoposide chemotherapy [17]. The average serum VEGF levels of the metastatic patients in this non-

controlled study, that was 495 pg/ml prior to therapy, dropped to 346 pg/ml 2 months after treatment. The serum VEGF values in our study, however, were 520 pg/ml prior to treatment and 299 pg/ml 3 months after the metronomic chemotherapy application.

The statistical analysis did not find significant differences, most likely due to the small number of patients that could be included in the study, as well as their heterogeneous distribution. However, the decrease in the average initial VEGF levels and those 3 months after in some patients receiving metronomic chemotherapy appears clinically significant. For example, the VEGF levels in C.C, a patient with breast cancer (stable disease) who responded to metronomic chemotherapy dropped from 660 to 410 pg/ml; VEGF level of H.T, a breast cancer patient who responded partially to metronomic chemotherapy, decreased from 280.2 to 128.5 pg/ml, and VEGF level in F.S, a small cell lung cancer patient, decreased from 898 to 197.5 pg/ml. However, the VEGF level in M.S, a breast cancer patient who did not respond to metronomic chemotherapy remained stable between 521.5 and 518.5 pg/ml, while the VEGF level of another breast cancer patient (G.B.), who progressed under metronomic chemotherapy rose from 450.5 to 676 pg/ml. A certain regression from 202 to 160.4 pg/ml was observed in H.U, a small cell lung cancer patient in progress under metronomic chemotherapy. There seems to be a trend of association between the patients' clinical data receiving metronomic chemotherapy and their VEGF levels. This aspect supports the idea that the activity of metronomic chemotherapy could be traceable prior to and after treatment, while it also appears to be worth a research with larger number of patients.

The decrease in the initial and 3-month later levels of VEGF in patients who received bevacizumab was significant ($p=0.002$). Likewise, a decrease in VEGF levels was also observed in patients who responded to bevacizumab therapy. Like with metronomic chemotherapy, the traceability of the bevacizumab activity using the pre- and post-therapy VEGF parameters seems possible. However, administration of the costly anti-VEGF therapy to patients with normal VEGF levels (150 ± 50 pg/ml) is questionable. These problems may possibly be overcome by means of a more strict

selection of patient subgroups where the anti-VEGF monoclonal antibody therapy would not be effective.

The development and formulation of new anti-VEGF therapies is continued. For example, anti-VEGF monoclonal antibody, soluble Flt-1, anti-KDR kinase (SU 5416 and SU 6668) and anti-KDR or anti-Flt-1 antibody are the anti-VEGF agents that have been used up until now [10]. The results of the present study suggest that the angiogenetic process in metastatic tumors could be traceable by simple and cost-effective serum VEGF level measurements. These measurements should be tried in all treatments and activity follow up studies that have an effect on the angiogenetic process including anti-VEGF monoclonal antibodies, metronomic therapies, thalidomide, lenalidomide and even some alternative/complementary methods said to target angiogenesis.

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The effects of sunitinib malate used in targeted therapy on the proliferation of HeLa cells *in vitro*

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Summary

Purpose: In this study, the antiproliferative and apoptotic effects of sunitinib (SU-11248, Sutent) which is used for targeted therapy was evaluated on HeLa cell line originated from human cervix carcinoma.

Methods: Three different doses of sutent ($D_1 = 1 \mu\text{M}$, $D_2 = 5 \mu\text{M}$, $D_3 = 10 \mu\text{M}$) were administered to cells for 72 h to determine the optimal dose.

Results: Increase in apoptotic index (AI), decrease in mitotic index (MI) of cells and slow down in proliferation rate were achieved at the dose level of $10 \mu\text{M}$, especially at 72 h. All these findings were statistically significant ($p < 0.001$). In addition, anaphase bridges and existence of tripolar metaphase cells that were observed at 72 h were possibly attributable to a chromosomal instability arising from shortening of telomere.

Conclusion: In this study, sutent effected cell kinetic parameters significantly. These results are consistent with other studies in the literature. In addition, anaphase bridges which were seen in mitosis preparations were interpreted as shortening or degradation of the telomere.

Key words: apoptotic index, cancer, mitotic index, sunitinib, sutent, targeted cancer therapy

Introduction

Cancer is an abnormal growth of cells caused by multiple changes in gene expression leading to dysregulated balance of cell proliferation and cell death and ultimately evolving into a population of cells that can invade tissues and metastasize to distant sites, causing significant morbidity and, if untreated, death of the host [1]. Chemotherapy is one of the conventional methods used for the treatment of cancer. Conventional chemotherapy, although directed toward certain macromolecules or enzymes, typically does not discriminate effectively between rapidly dividing normal cells and tumor cells, thus leading to several toxic side effects [2]. Therefore in recent years targeted therapies have been started to develop. Targeted cancer therapies are drugs or other substances that block the growth and spread of cancer by interfering with specific molecules involved in tumor growth and progression [3]. One of the drugs used in targeted therapies is sunitinib (SU-11248, sunitinib). Sunitinib is a small-molecule receptor tyrosine kinase inhibitor that inhibits cellular signaling of multiple targets, approved for advanced renal cell carcinoma and imatinib-resistant or imatinib-intolerant gastrointestinal stromal tumors [4,5]. Sunitinib inhibits at least 8 receptor protein-tyrosine kinases including vascular endothelial growth factor receptors 1–3 (VEGFR1–VEGFR3), platelet-derived growth factor receptors (PDGFR α and PDGFR β), stem cell factor receptor (Kit), Flt-3, and colony-stimulating factor-1 receptor (CSF-1R) [6].

In this study we investigated the antiproliferative and apoptotic effects of sunitinib on HeLa cell line originated from human cervix carcinoma. Three different doses of sunitinib were administered to the cells in order to determine the optimal dose from 12 to 72 h. The effects of sunitinib on the growth rates of HeLa cells were evaluated with the WST-1 assay kit, while the MI and AI were determined with using light and fluorescence microscope, respectively.

Methods

Cell culture

The HeLa cell line used in this study was obtained from European Cell Culture Collection (ECACC). Cells were cultured in Medium-199 (M-199, Sigma, USA)

containing 10% fetal bovine serum (FBS, Gibco Lab), 100 μ g/ml streptomycin (Streptomycin sulphate, I. E. Ulugay), 100 IU/ml penicillin (Pronapen, Pfizer), amphotericin B (Sigma, USA) and 2 mM glutamine at 37 °C in humidified atmosphere of 5% CO₂. The pH of the medium was adjusted to 7.4 with NaHCO₃.

Drug doses

Sunitinib concentrations that were used in the present study were determined based on previous *in vitro* and clinical studies. First, 1 mM stock solution was prepared with M-199 supplemented with 10% FBS. Three different doses were obtained by dilution of the stock solution and were determined as dose 1 (D₁) = 1 μ M, dose 2 (D₂) = 5 μ M and dose 3 (D₃) = 10 μ M. HeLa cell cultures were exposed to the 3 doses for 12, 24, 48 and 72 h.

Determination of cytotoxic activity with WST-1 (mitochondrial dehydrogenase enzyme activity)

The effects of sunitinib on the growth rates of HeLa cells were evaluated with the WST-1 assay kit (Roche). The WST-1 assay was applied to identify the cytotoxicity of sunitinib after 12, 24, 48 and 72 h of exposure. For WST-1 assay cells were cultured in 96-well plates in a final volume of 200 μ l/well culture medium in a humidified atmosphere. Twenty μ l of the cell proliferation reagent WST-1 were added to each well. Cells were incubated for 4 h in a humidified atmosphere. At the end of this period, cells were shaken thoroughly for 1 min on the shaker. Then, absorbance of the samples was measured against a background control as a blank using a ELISA reader (μ Quant, Bio-Tek Instruments Inc, USA) at 420–480 nm [7].

Determination of optimal dose with mitochondrial dehydrogenase enzyme activity analysis

In this examination 3 different doses of sunitinib (D₁ = 1 μ M, D₂ = 5 μ M, D₃ = 10 μ M) were applied to HeLa cell culture for 72 h and the cytotoxic effects of these doses were evaluated with mitochondrial dehydrogenase enzyme activity analysis (WST-1 assay). As a result of this application, absorbance values were measured for each dose and the most effective dose for HeLa cell culture was determined.

Mitotic index analysis

The MI was studied using the Feulgen method [8,9]. Before the cells were stained with Feulgen, they were prepared with 1 N HCl at room temperature for 1 min and then hydrolyzed with 1 N HCl for 10.5 min at 60°C. After slides were stained with Feulgen, they were rinsed for a few minutes in distilled water and stained with 10% Giemsa stain solution at pH 6.8 for 3 min and washed twice in phosphate buffer. After staining, the slides were rinsed in distilled water and then were air-dried. Finally, the MI was calculated by counting metaphases, anaphases and telophases for each control group which was not exposed to sunitinib and the experimental groups which were exposed to D₃ dose (10µM) of sunitinib for 12, 24, 48 and 72 h. At least 3,000 cells were examined using light microscope from each slide to determine the MI.

Apoptotic index analysis

The AI (the percentage of cells undergoing apoptosis) was studied using fluorescence microscope. For the determination of the AI, cells were fixed with methanol and stained with 4'-6 diamidine-2 phenylindol

(DAPI). Following extensive washing in phosphate-buffered saline (PBS), slides were scored under fluorescence microscope. For evaluation of the AI at least 100 cells were counted for the control and each experimental group.

Statistics

Values of proliferation rate, MI and AI were evaluated relative to controls and to each other. For this reason, values obtained from all experimental groups were analyzed using one-way ANOVA test. The significance between control and experimental groups was determined by DUNNETT's test and the significance between experimental groups was determined by Student's t-test.

Results

Determination of optimal dose with mitochondrial dehydrogenase enzyme activity analysis The absorbance values of each dose for 72 h are shown in Table 1. All the differences between control and experimental groups were statistically significant ($p < 0.001$). In addition, significant differences among the experimental groups were noted ($p < 0.001$) (Figure 1). Seventy-two hours after drug exposure, mitochondrial dehydrogenase enzyme activity values were 66% for D₁, 46% for D₂ and 23% for D₃ compared with the control group which was considered 100% (Figure 2).

Determination of cytotoxic activity with WST-1 (mitochondrial dehydrogenase enzyme activity analysis)

Amongst the three different sunitinib doses administered to the cells for 72 h, D₃ dose of sunitinib that inhibited the cell proliferation effectively compared to the other

Table 1. Absorbance values of mitochondrial dehydrogenase enzyme activity of HeLa cells treated with 3 different doses of sunitinib (D₁ = 1µM, D₂ = 5µM, D₃ = 10µM) (±SD)

Groups	Absorbance values (450-690 nm)
Control	472.231x10 ⁻³ * ± 0.32
D ₁	312.231x10 ⁻³ ± 0.29
D ₂	217.423x10 ⁻³ ± 0.43
D ₃	111.625x10 ⁻³ ± 0.52

SD: standard deviation. *Significantly different ($p < 0.01$)

Table 2. Absorbance values of mitochondrial dehydrogenase enzyme activity of HeLa cells treated with D₃ dose of sunitinib for 0-72 h (±SD)

Experimental groups Time of exposure (hours)	Absorbance values (450-690 nm)	
	Control group	D ₃
0	259.354x10 ⁻³ * ± 0.27	258.243x10 ⁻³ ± 0.22
12	321.230x10 ⁻³ ± 0.19	212.342x10 ⁻³ ± 0.28
24	398.256x10 ⁻³ ± 0.33	194.784x10 ⁻³ ± 0.31
48	429.670x10 ⁻³ ± 0.41	168.298x10 ⁻³ ± 0.47
72	472.231x10 ⁻³ ± 0.32	111.625x10 ⁻³ ± 0.52

SD: standard deviation. *Significantly different ($p < 0.01$)

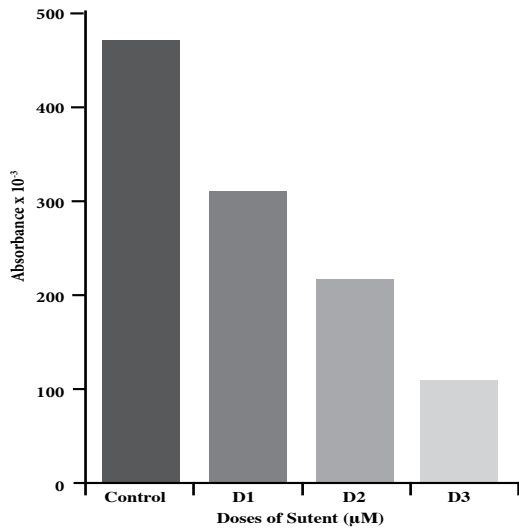


Figure 1. Absorbance values of mitochondrial dehydrogenase enzyme activity of HeLa cells treated with three different doses of sunitinib ($D_1 = 1\mu\text{M}$, $D_2 = 5\mu\text{M}$, $D_3 = 10\mu\text{M}$) for 72 h (450-690 nm) ($p < 0.01$).

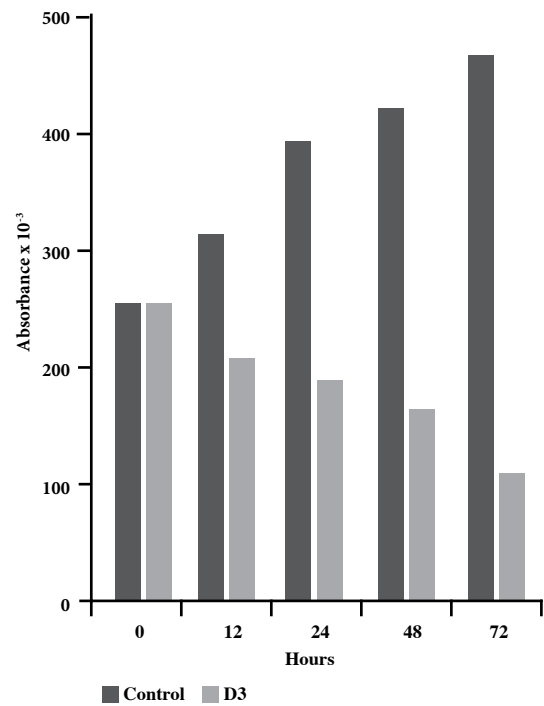


Figure 3. Absorbance values of mitochondrial dehydrogenase enzyme activity of HeLa cells treated with D_3 dose of sunitinib ($D_3 = 10\mu\text{M}$) for 0-72 h (450-690 nm) ($p < 0.01$).

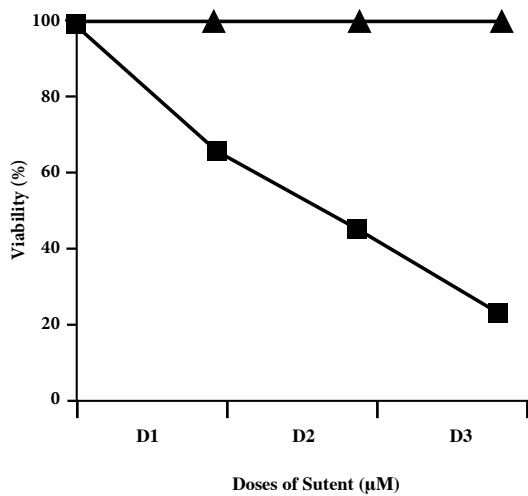


Figure 2. Percent viability values of HeLa cells treated with three different doses of sunitinib ($D_1 = 1\mu\text{M}$, $D_2 = 5\mu\text{M}$, $D_3 = 10\mu\text{M}$) for 72 h (the horizontal line shows the control group).

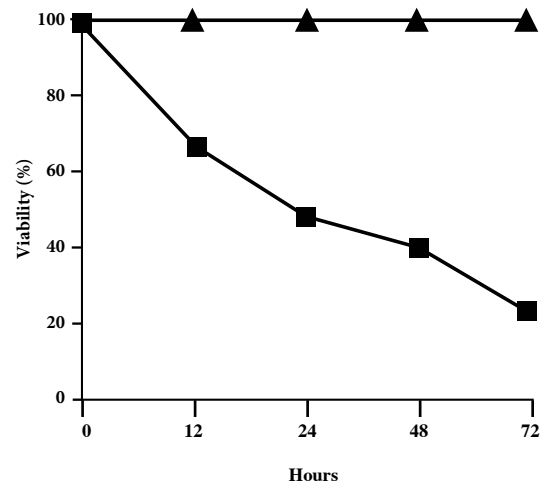


Figure 4. Percent viability values of HeLa cells treated with D_3 dose of sunitinib ($D_3 = 10\mu\text{M}$) for 0-72 h (the horizontal line shows the control group and the dropping line the D_3 dose).

Table 3. Mitotic index values of HeLa cells treated with D_3 dose of sunitinib ($D_3 = 10\mu\text{M}$) for 0-72 h (\pm SD)

Experiment groups Time of exposure (hours)	Mitotic index values (%)	
	Control	D_3
12	7.62 \pm 0.24*	5.00 \pm 0.12
24	7.21 \pm 0.51	3.44 \pm 0.37
48	5.28 \pm 0.22	2.42 \pm 0.13
72	5.14 \pm 0.21	1.10 \pm 0.14

SD: standard deviation. *Significantly different ($p < 0.01$)

doses was applied to the cells at 0,12,48 and 72 h.

After treatment of HeLa cell culture with D_3 , absorbance values of the control and experimental groups were measured for each hour (Table 2). Statistical analysis showed significant difference between control and experimental groups at 12-72 h ($p < 0.001$) (Figure 3).

According to the absorbance values, while viability of the control group was considered 100%, the viability values of the experimental groups compared with the control group were 66% in 12 h, 48% in 24 h, 39% in 48 h and 23% in 72 h (Figure 4).

According to the mitochondrial dehydrogenase enzyme activity analysis which was performed with administration of sunitinib for 0-72 h it was concluded that the viability of HeLa cells was significantly decreased in a time-dependent manner ($p < 0.001$).

Apoptosis

Morphological evaluation

The apoptotic morphological changes were shrinkage and blebbing of the cell membrane, and nuclear condensation, cell deformation, formations of apoptotic bodies and nuclear breaks into fragments. These apoptotic morphological changes were detected under light and fluorescence

microscope on HeLa cells treated with D_3 of sunitinib for 12, 24, 48 and 72 h.

Apoptotic index

For 0-72 h after administration of D_3 dose of sunitinib which was the most effective dose compared to other doses, approximately 200 cells were counted for both control and experimental groups. AI was 3% for the control, 34% for D_1 , 58% for D_2 and 67% for D_3 for the experimental groups (Figure 5). All the differences between control and experimental groups were statistically significant ($p < 0.001$). In addition, a significant difference was noted among experimental groups ($p < 0.005$). AI values of D_3 dose for 0-72 h are shown in Figure 5. Statistically, there was a significant difference between the control and experimental groups ($p < 0.001$).

Mitosis

Morphological evaluation

Late prophase, metaphase, anaphase and telophase of mitosis in HeLa cells were evaluated with Feulgen method and Giemsa staining under light microscope. Administration of D_3 of sunitinib at 72 h caused tripolar metaphase and a plenty of anaphase bridges, a phenomenon that doesn't occur in normal cell division.

Mitotic index

For 0-72 h after administration of D_3 dose of sunitinib which was the most effective dose compared to other doses, 3000 cells were counted for both the control and experimental groups.

MI values of D_3 dose are shown in Table 3. The difference was significant between the control and experimental groups ($p < 0.001$). In addition, statistically significant difference was noted among all experimental groups ($p < 0.001$). The optimal dose of sunitinib was D_3 (MI 1.10%). MI values were significantly different between the control and experimental groups ($p < 0.001$).

Discussion

In this study, the cytotoxic effects of sunitinib were evaluated with parameters of cell kinetics including mitochondrial dehydrogenase enzyme activity analysis, AI and MI.

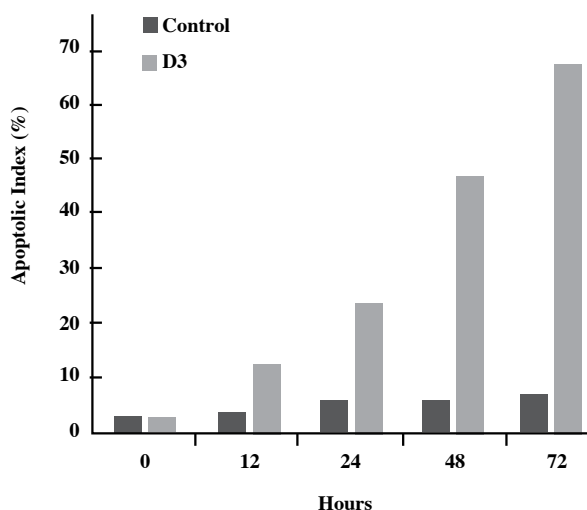


Figure 5. Apoptotic index values of HeLa cells treated with D_3 dose of sunitinib for 0-72 h and control ($p < 0.01$).

Sutent exerts cytotoxic activity in breast, thyroid and colorectal cancer as well as in metastatic gastrointestinal tumors and metastatic renal cell carcinoma [10-12].

Administration of sutent to human cord endothelial cells brings about apoptosis [13]. In a study which examined different methods of tumor therapy, it was supposed that inhibition of angiogenesis can be an effective anticancer therapy [14].

An *in vitro* study indicated that sutent is an effective drug derived from indole compounds. Sutent inhibited the proliferation of TPC-1 cells in a dose-dependent manner (IC_{50} 224 nM). It targeted tumor cell proliferation and survival directly with suppression of receptor tyrosine kinases (RTK) such as PDGFR, VEGFR, KIT and FLT3. This study showed that sutent was an inhibitor of RET/PTC oncoprotein with IC_{50} 224 nM and was more advantageous because of low toxicity, antiangiogenic activity and oral intake compared with other compounds which were developed for RET/PTC associated papillary thyroid carcinoma (PTC) therapy [15].

It was shown that 10 μ M of sutent have antiangiogenic activity on U87MC glioma cells and this high dose is not toxic for the healthy glial cells [16].

In our investigation, the optimal cytotoxic dose was 10 μ M. This dose was applied for 72 h to the cells. Mitochondrial dehydrogenase enzyme activity was detected and as a result, while viability was 100% for the control group, viability of the experimental groups dropped to 23%.

In a study with preclinical evaluation of sutent, based on data derived from breast cancer, this drug exerted intense antiproliferative activity alone or combination with conventional cytotoxic agents (5-fluorouracil, doxorubicin) [17].

In a clinical investigation with sutent administered to patients with gastrointestinal stromal tumor, apoptosis increased 6-fold in all patients receiving the drug compared to pre-treatment levels [18].

In an *in vitro* investigation, it was demonstrated that sutent suppresses cellular proliferation and increases apoptosis in a dose-dependent manner in MV4-11 cell line (IC_{50} 1-10 nM) [19].

In another study which was done on U87 and M059K glioma cells, it was observed that cell pro-

liferation decreased after 48 h exposure to sutent. The proliferation of these cell lines was inhibited by 95% after 10 μ M of sutent. In addition, a significant increase in apoptosis was observed after 24 h of exposure of these cell lines to sutent [20].

Phosphorylation of CSF-1R receptor that belongs to RTK family and is expressed by NIH3T3 cells, was inhibited with IC_{50} 50-100 nM of sutent. An *in vivo* study on mice, 40 mg/kg/day of sutent for 21 days inhibited tumor growth in bone by 64%. Depending on these findings, it was concluded that this drug can be used as an effective agent for prevention of bone metastasis in breast cancer [21,22].

In an *in vitro* study done on 5637 and TCC-SUP cell lines of human invasive transitional cell carcinoma, sutent blocked cell proliferation at a dose of IC_{50} 9.9 μ M and IC_{50} 7.5 μ M, respectively [23].

In our study, administration of D_3 (10 μ M) of sutent for 72 h increased the AI to 67% vs. 3% in the control group ($p < 0.001$). This increase was thought as an important finding for inhibition of tumor growth.

Also, MI was 1.1% for the experimental groups and 5.14% for the control group after administration of sutent at D_3 for 72 h. These data suggested that the reduction in the number of cells in mitosis due to apoptotic cell deaths brought about the decrease of MI. Again, the decrease in the percentage of living cells with administration of D_3 dose for 72 h seems to support this opinion.

Determination of telomerase activity in tissues with the development of the TRAP method allowed the investigation of the expression of telomerase in a large number of cancers. Studies show that telomerase is the most common determinant of cancer; about 85% of malignant tumors display telomerase activity, suggesting that telomerase is a very important indicator in the diagnosis of cancer [24].

In our investigation, MI studies showed a relatively large number of cells with anaphase bridges and a small number of cells with tripolar metaphase with administration of D_3 dose for 72 h. Relative to this unexpected finding, some studies had shown that anaphase bridges occur due to telomere shortening or degradation [25-29]. Also, the authors of a study reported a complete relationship between reduction

of telomere length and frequency of endogenous bridges [30].

In the present study, changes in the cell cycle of HeLa cells caused by sunitinib were investigated and, to our knowledge, this is the first investigation on this topic. Administration of sunitinib to HeLa cell culture with optimal dose (10 μ M), especially for 72 h, caused significant increase of AI and decrease of MI. Also the existence of anaphase bridges and tripolar metaphase in 72 h, but not in previous hours, suggested that there was a chromosome instability caused by telomerase shortening. This assumption can lead to new investigations about the use of sunitinib for telomerase-targeted therapy.

Acknowledgment

This work was supported by the Research Fund of the University of Istanbul, Project no. 3852.

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Acute infusion reactions to chemotherapeutic drugs: a single institute experience

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Summary

Purpose: Treating cancer often involves the use of chemotherapeutic agents. Due to the growing incidence of cancer worldwide and the expanding number of treatment options, it is important to understand the risks of adverse events associated with these treatments. In this study, we monitored the occurrence of acute infusion reactions in an outpatient chemotherapy center from April 2011 to April 2012.

Methods: For patients who developed infusion reactions, the causative drug, the dose and number of treatments received, the onset time of the reaction, the duration of the reaction, blood pressure, pulse, level of oxygen saturation during the reaction, and other symptoms were recorded. The severity of reactions was determined in accordance with NCI toxicity criteria. A reaction was considered as grade 1-2 (mild-moderate) if the patient experienced flushing, rash, fever, tremor, dyspnea, rigor, and mild hypotension. Symptoms such as severe hypotension, bronchospasm, cardiac dysfunction and anaphylaxis, requiring therapeutic intervention, were classified as severe, grade 3-4 reactions.

Results: Of the 2213 patients receiving chemotherapy during the study period, 138 (62%) developed an infusion reaction to the treatment. Among 138 patients most commonly treated types of carcinoma included breast (39.2%), lung (17.8%), colorectal (10%), and ovarian (8.5%) cancers. Docetaxel administration resulted in the largest number of infusion reactions, though most reactions were mild to moderate and did not require the cessation of treatment. Patients with mild to moderate reactions (89.2%) were able to continue treatment, while those who developed severe reactions (10.8%) could not continue treatment with the same agent.

Conclusion: Although severe reactions are rare, the incidence of mild to moderate reactions against taxanes, platinum compounds, and monoclonal antibodies is quite high. Clinical symptoms do not vary widely among the agents, though the onset time of symptoms does vary. While reactions against platinum agents were of type 1 anaphylactic reactions, reactions against taxanes and monoclonal antibodies during the first infusion and in the following minutes suggest the activation of different mechanisms.

Key words: acute adverse reaction, chemotherapy, infusion, safety

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Received: 20/05/2012; Accepted: 18/06/2012

Introduction

Cancer patients can develop unexpected adverse reactions to the administered chemotherapeutic agents, which differ from the known toxicities of these drugs. These infusion reactions have been shown to occur with a wide range of drugs [1]. It is difficult to predict the reactions that an individual may experience upon exposure to a drug. Infusion reactions range from mild to life-threatening [2,3].

Infusion reactions are classified as standard infusion reactions (SIR) or anaphylactic reactions. Although clinical findings often overlap, the symptoms of SIR typically include fever, tremor, flushing, dyspnea, itching, and changes in heart rate and blood pressure. In contrast, anaphylactic reactions are characterized by urticaria, sudden nasal congestion, zonesthesia, changed voice due to laryngeal edema, shortness of breath, wheezing, hypotension, and loss of consciousness.

The National Cancer Institute (NCI) classifies hypersensitivity reactions from grade 1 to grade 5. In grade 1 reactions, temporary flushing and rash are observed, and fever is $<38^{\circ}\text{C}$. In grade 2, rash, hives, dyspnea, and fever $>38^{\circ}\text{C}$ are also observed. In grade 3 reactions, hives that require parenteral intervention and allergy-related edema and hypotension are observed. Grade 4 reactions are defined as life-threatening anaphylaxis, and grade 5 reactions result in death [4].

The pathogenetic mechanisms of infusion reactions differ among different agents, and are not well-understood [5]. Most reactions that occur with the use of standard chemotherapeutic agents include type 1 hypersensitivity reactions [1,6,7]. True type 1 reactions are caused by the IgE mediated release of histamines, leukotrienes, and prostoglandins from mast cells in tissue and basophils in peripheral blood [7,8]. Reactions related to platinum-based compounds such as carboplatin and oxaliplatin are considered type I IgE-mediated reactions [9,10].

Metabolites of some chemotherapeutic agents may cause anaphylactic reactions by directly affecting mast cells and basophiles without the mediation of IgE [1]. Taxanes (docetaxel and paclitaxel) in particular can lead to clinical manifestations similar to type 1 hypersensitivity reactions [9,10].

In this study, we determined the incidence and characteristics of infusion reactions that developed

during treatments with chemotherapeutic drugs and monoclonal antibody therapies.

Methods

Among patients who were treated in the outpatient chemotherapy unit at the Adana Baskent University, Medical Oncology department between April 2011 and April 2012, those who developed infusion reactions were enrolled in the study. All participants provided informed consent before enrollment. The study was approved by the ethics committee at Baskent University Adana hospital. In the outpatient unit, a median of 970 patients (range 845-1100) receive chemotherapy each month. The most common cancer types in our unit are lung (15.2%), breast (21%), and colorectal cancer (8.4%).

For patients who developed infusion reactions, the causal drug, the dose and number of treatments received, the onset time of the reaction, the duration of the reaction, blood pressure, pulse, level of oxygen saturation during the reaction, and other symptoms were recorded. The severity of reactions was determined in accordance with NCI toxicity criteria. A reaction was considered as grade 1-2 (mild-moderate) if the patient experienced flushing, rash, fever, tremor, dyspnea, rigor, and mild hypotension. Symptoms such as severe hypotension, bronchospasm, cardiac dysfunction and anaphylaxis, requiring therapeutic intervention, were classified as severe, grade 3-4 reactions.

Before the use of a monoclonal antibody, each patient was given dexamethasone (16 mg) and premedication with ranitidine (50 mg) and pheniramine (45.5 mg) i.v. infusion as antihistaminics. Before the administration of cytotoxic drugs, dexamethasone (16 mg), ranitidine (50 mg), and ondansetron (8 mg) i.v. were routinely given. Any patient who developed an infusion reaction was additionally given prednisolone (40 mg), pheniramine (45.5 mg) and, in the presence of shortness of breath and cyanosis, an inhaler solution of albuterol sulphate. In patients thought to have grade 1-2 reactions, the treatment drug was resumed at a lower infusion rate after the resolution of symptoms. In patients with grade 3-4 reactions, use of the treatment drug was not resumed. Alternative drug treatments were explored in these patients.

Results

In this study we monitored the occurrence of acute infusion reactions in the outpatient chemotherapy center from April 2011 to April 2012. Of 2213 patients receiving chemotherapy during the study period 138 (6.2%) developed an infusion reaction to the therapeutic agents administered. There were 94 (68.1%) female and 44 (31.9%) male patients with age range 32-79 years (median 56). Among the 138 patients, the most common cancer type was breast (39.2%), followed by lung (17.8%), colorectal (10%), ovarian (8.5%), and all others (28.5%). Of these reactions, 58.6% occurred during infusion of taxanes (docetaxel and paclitaxel), 23% occurred during the use of platinum agents (carboplatin, oxaliplatin, cisplatin), and 18% occurred during infusion of monoclonal antibodies (trastuzumab, rituximab).

Table 1 shows the percentages of allergic reactions developed by each drug.

Table 2 summarizes the number of subjects, the number of the cycles given, and the number and grade of reactions for patients during the 12-month study period.

While 64 of 67 (95.5%) patients who had developed allergies against docetaxel showed grade 1-2 reactions, 3 (4.5%) patients developed grade 3-4 reactions. Most (54 of 67; 80.5%) patients experienced the reaction during the first cycle, while 13 (19.5%) patients experienced the reaction during cycles 2-3. In all patients, the reactions to docetaxel occurred

within the first 1-5 min of the infusion. Patients who developed reactions were given prednisolone (40 mg) and pheniramine (45.5 mg). Therapy had to be discontinued in one patient with a grade 3-4 reaction. In all other patients, symptoms resolved and therapy was resumed. Of the patients who developed allergy to docetaxel, 53 (79.1%) did not receive standard premedication with oral prednisolone 12 and 1 hour prior to receiving chemotherapy. In contrast, 14 (20.9%) patients developed allergic reaction despite the standard premedication.

Thirty-two patients had an allergic reaction to platinum agents. None of the 18 patients with carboplatin allergy developed the reaction during the first cycle. Carboplatin reactions that developed during the third and following cycles were grade 1-2 in 12 patients (66.6%) and grade 3-4 in 6 (33.4%) patients.

Table 1. Distribution and percents of the allergic reactions by drug

<i>Drugs</i>	<i>Allergic reactions</i> <i>N</i>	<i>%</i>
Docetaxel	67	48.5
Paclitaxel	14	10.1
Carboplatin	18	13.0
Oxaliplatin	10	7.2
Cisplatin	4	2.8
Trastuzumab	14	10.1
Rituximab	11	7.9
Total	138	100

Table 2. Percents of reactions along with the total number of cycles given and the number of patients

<i>Drug</i>	<i>Number of patients who received the drug</i>	<i>Number of cycles given</i>	<i>Number of reactions</i> <i>N</i>	<i>Grade 1-2 reactions</i> <i>N</i>	<i>Grade 3-4 reactions</i> <i>N</i>	<i>Percents within subject %</i>
Docetaxel	242	973	67	64	3	27.6
Paclitaxel	144	532	14	14	-	9.7
Carboplatin	162	814	18	12	6	11.1
Oxaliplatin	62	708	10	7	3	16.1
Cisplatin	183	1360	4	2	2	2.1
Trastuzumab	216	1872	14	13	1	6.4
Rituximab	61	416	11	11	-	18.0
Total	1070	-	138	123	15	-

In patients with grade 3-4 reactions, carboplatin therapy was not continued. In patients with grade 1-2 reactions, therapy was continued after resolution of symptoms. The adverse reaction occurred within the first 15 min in 6 patients and between 40th and 60th min in the others.

Of 4 patients who developed cisplatin allergy, 2 experienced grade 1-2 reactions after the 4th cycle, and 2 grade 3-4 reactions after the 3rd cycle of chemotherapy. One patient required intubation due to serious bronchospasm, hypotension, and respiratory arrest. Adverse reactions were observed 40 min after the initiation of therapy in one patient and 60-90 min after initiation of therapy in the others.

Of 10 patients who developed oxaliplatin allergy, one patient developed a reaction in the 2nd cycle, while the others developed the reaction during the 4th through 6th cycles. The time of onset of the reactions was 30-45 min after initiation of therapy. Seven patients developed grade 1-2 reactions and continued therapy after the resolution of symptoms. However, the other patients discontinued therapy due to grade 3-4 reaction. Upon development of the same grade reactions during repeated administrations, therapy was discontinued.

Rituximab, a monoclonal antibody, led to an infusion reaction in 11 (7.9%) patients. The reaction developed after the first infusion in 9 patients and after the second infusion in 2 patients. The time of onset of the reactions was 40-70 min after the initiation of infusion. Generally, grade 1-2 reactions, characterized by fever, tremor, nausea, headache, and abdominal pain were observed. None of the patients experienced grade 3-4 reactions. Therapy could be continued in all patients.

In 14 patients who received trastuzumab, infusion-related reactions were recorded. One patient developed respiratory and cardiac arrest following symptoms of severe bronchospasm, hives, and hypotension within 30 min of starting the infusion. After intervention, the patient was intubated and monitored in the intensive care unit. After 24 hours, he was extubated. Upon improvement of his general status, he was discharged. The remaining 13 patients developed grade 1-2 reactions between 30 and 60 min fol-

lowing the first infusion. In 13 patients, therapy could be continued after resolution of symptoms. No adverse events were seen with repeated infusions.

In total, 15 patients (10.8 %) developed grade 3-4 reactions and could not continue therapy. In contrast, 123 patients (89.2 %) showed grade 1-2 reactions and could continue therapy.

Discussion

In cancer treatment, hypersensitivity reactions may develop with the use of nearly all types of systemic agents (cytotoxics and monoclonal antibodies). With the worldwide increasing incidence of cancer, the use of these drugs has also increased significantly. In the literature, the majority of infusion reactions (95%) have been reported to be of grade 1-2, or mild to moderate [11]. In our study, the percentage of severe reactions was 10.8 %. The majority of these reactions occurred against platinum compounds, while only 3 severe reactions were observed against taxanes.

Taxanes are very commonly used in both metastatic and adjuvant therapy protocols, either as a single-agents or in combined regimens. Taxanes may cause both SIR and anaphylactic reactions. Chromophore, which is present in the formulation of paclitaxel, and polysorbate 80, which is present in the formulation of docetaxel, are a major cause of these reactions. Adverse reactions caused by paclitaxel seem to be caused by complement activation, mast cell/basophil activation, and classical IgE-mediated anaphylaxis [12]. The pathogenesis of reactions caused by docetaxel has not been fully characterized [13].

The percentage of reactions that resulted from docetaxel has been shown to be 5-20%, while the percentage of severe reactions, despite standard premedication, was 2% [14]. Nearly half (45%) of the allergic reactions seen in our outpatient unit were related to the use of docetaxel, and the percentage of reactions to docetaxel was 26%. In our study, nearly 80% of the patients developed this reaction during the first docetaxel infusion, within the first 1-5 min of infusion. These rates are consistent with the literature [14]. Although the reaction to docetaxel is very commonly observed in clinical practice, it is important to note that most patients experienced grade 1-2

reaction but were able to continue treatment. In our unit, the percentage of the reaction against paclitaxel was 10 %, consistent with the range of 8-45% that has been reported [15].

The incidence of any grade reaction to platinum compounds has been reported to be 12-20% [9,10,16,17]. In our unit, platinum agents (carboplatin, oxaliplatin and cisplatin) produced an overall reaction rate of 29.3%. The highest rates of grade 3-4 reactions were caused by platinum agents.

The most commonly used platinum agent that caused reactions was carboplatin. Carboplatin is most commonly used to treat ovarian and lung cancer. A study reported that the incidence of any type of reaction to carboplatin was 12% [17]. In the same study, 27% of the patients who received 7 or more cycles with carboplatin developed reactions, compared to approximately 1% in those who received less than 7 cycles [17]. Tamiya et al. [18] determined that the overall incidence of allergic reactions in lung cancer patients treated with cisplatin or carboplatin was 1.96%. Furthermore, in this study, a direct relationship was found between the number of platinum therapy cycles and the occurrence of hypersensitivity reactions. In the literature, 50% of patients who developed an infusion reaction against platinum agents went on to develop reactions to repeated drug administrations, despite premedication [20,22-24]. A fatal case related to the use of cisplatin was reported [25]. Cross-allergy exists between cisplatin and carboplatin, but its incidence is not known [26]. Therefore, if a platinum compound will be re-used, desensitization is recommended [27-29].

Due to the increasing use of carboplatin as both a first- and second-line therapy for ovarian cancer, the incidence of allergic reactions also increased. Polyzos et al. reported that the incidence of allergic reactions in ovarian cancer patients treated with carboplatin was 16%, mostly after the 4th course [9]. In this study, the majority of reactions were mild to moderate and the rate of severe reactions was 6.1%. No patient with severe reaction continued treatment [9]. In our study, the rate of carboplatin allergy was 11.1%, 50% of which were grade 3-4 reactions that resulted in discontinuation of treatment. In a retrospective study, it

was reported that increasing the duration of carboplatin infusion from 30 min to 3 hours decreased the number and severity of reactions [19].

With the growing use of oxaliplatin in the FOLFOX and XELOX regimens for the treatment of colorectal cancer, the incidence of hypersensitivity reactions has incrementally increased. Although there are more case reports about oxaliplatin allergy, a study published in 2006 showed that 15% of 108 patients showed an allergic response. The rate of severe reactions was 2.2% over 5 years. When oxaliplatin was readministered to 14 patients who developed reactions, recurrent development of the allergy was seen [20]. In a subsequent large-scale study, 308 of 1224 patients (25%) developed oxaliplatin allergy. Most reactions were observed after the first 5 cycles. The percentage of grade 1-2 reactions was 23%, while the percentage of grade 3-4 was 37% [9,16,20,21]. In our study, 16.1% of the patients showed a reaction to oxaliplatin. Three patients (30% of the reacting ones) could not continue therapy due to a grade 3-4 reaction after the 4th and 5th cycles.

While infusion reactions to monoclonal antibodies are typically seen within the first 30 to 120 min after initiation of infusion, the majority of reactions occur during the first and second infusion [11]. Reactions are generally mild-moderate and rarely show a fatal course. Monoclonal antibodies cause both SIR and anaphylactic reactions, but anaphylaxis is rarely seen. With rituximab, more than 50% of the reactions were seen during the first infusion. These reactions were proportional to the levels of CD20 cells in the blood. In our study, the rate of rituximab reactions was 18.7%. Since the reactions were grade 1-2, no drug discontinuation was needed.

The incidence of reaction during the first infusion of trastuzumab is 20-40% [30,31], with 0.3% of the reactions are grade 3-4. The incidence and severity of adverse reactions to trastuzumab is lower than that caused by rituximab [32]. In our unit, the rate of reaction caused by trastuzumab (6.4%) was lower compared with rituximab (18%), and one patient required intubation due to a grade 3-4 reaction during the first infusion of trastuzumab. She was discharged after being extubated. Other patients with grade 1-2

reactions could continue to use the drug safely.

The incidence of infusion reactions due to the administration of cetuximab varies by geographic region. While two studies conducted in Europe showed an incidence of grade 3-4 reactions as 2.5% and 3.5%, respectively [33,34], the incidence of grade 3-4 reactions increased to 20% in the southeast region of the USA [35]. No cetuximab allergy was encountered during our study.

Consequently, although severe reactions are rare, the incidence of mild to moderate reactions against taxanes, platinum compounds, and monoclonal antibodies is quite high. Clinical symptoms do not vary widely among the agents, though the onset time of symptoms does vary. While reactions against platinum agents was accounted for type 1 anaphylactic reactions, in contrast, reactions against taxanes and monoclonal antibodies during the first infusion and in the following minutes suggests the presence of different mechanisms.

It is important to accurately determine the grade of adverse reactions. While mild-moderate reactions (grade 1-2) tend to present with fever, skin rash, flushing, tremor, itching, and dyspnea, severe reactions (grade 3-4) tend to present with serious hives, bronchospasm, wheezing, zonesthesia, and voice alterations. Mild-moderate reactions can be controlled by temporary discontinuation of treatment and administration of symptomatic supportive therapy.

Although the same drug can be continued after complete resolution of the symptoms, subsequent administrations should include premedication, decrease of the infusion rate, and/or a desensitization protocol. In severe reactions, the infusion should be discontinued and supportive therapy should be initiated. In such events, the drug should be changed if possible, and if it is necessary to continue with the same drug, a desensitization program should be applied.

Acknowledgement

This study was approved by the Baskent University Institutional Review Board (projet no : KA12/24) and supported by the Baskent University Research Fund.

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Evaluation of the role of radiotherapy in the management of dermatofibrosarcoma protuberans

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Summary

Purpose: The aim of this study was to evaluate the role of radiotherapy (RT) in the management of dermatofibrosarcoma protuberans (DFSP).

Methods: Twenty-eight patients treated with RT for DFSP between 1974 and 2012 at Gulhane Military Medical Academy (GMMA) Radiation Oncology Department were retrospectively evaluated. Twenty-five out of 28 patients (89%) received postoperative RT and 3 received definitive RT alone. In the 25 patients receiving postoperative RT, the type of surgical excision was limited excision in 5 patients and wide excision in the remaining 20. Median RT dose was 63.21±3.7 Gy (range 50-70).

Results: At a median follow-up of 5 years, 5-year overall survival (OS) for the whole patient group was 93%. No relationship was determined between the total delivered RT dose and OS. The 5-year OS of the 10 female patients was 90% whereas it was 94% for the 18 male patients (p>0.05). Five-year disease-free survival (DFS) for the patients undergoing wide excision with RT vs. those undergoing limited excision with RT was significantly superior (p<0.05) in patients treated with wide excision and RT.

Conclusion: RT is an effective treatment option for DFSP patients with positive postoperative margins, recurrent disease and selected inoperable cases.

Key words: dermatofibrosarcoma protuberans, limited excision, radiotherapy, wide excision

Introduction

DFSP is a soft tissue neoplasm with a very limited metastatic potential, however, it has a high propensity for local invasion and recurrence.

This monoclonal dermal fibroblast-originated sarcoma, mostly arising within the dermis, has an incidence of 0.8 cases per million people per year [1]. Lesions are mostly low-grade with indolent growth, rarely metastasizing through lymphatic or hematogenous routes but frequently recurring locally [1,2]. Lesions with high-grade fibrosarcoma components tend to behave more aggressively [3,4]. Diagnosis is often delayed until the lesions reach a large size. Trunk, head and neck, and proximal extremities comprise the most common locations for DFSP, presenting with red or pink dermal painless plaques. DFSP occurs more commonly in men, typically manifesting in the fourth decade of life. Horizontal dermal persisting growth pattern is typical prior to deep extension and fixation to the subcutaneous tissue with nodular growth. Core needle or incisional biopsy along with magnetic resonance imaging (MRI) to assess invasion depth are important steps of the work-up. DFSP is staged according to the guidelines of American Musculoskeletal Tumor Society as IA or IB, low-grade IA not extending beyond the subcutaneous compartment and low-grade IB involving fascia or muscle [4]. Surgery with clear margins constitutes the mainstay of treatment for DFSP. RT may be used either before or after surgery with regard to certain characteristics. Patients with large or unresectable tumors often receive preoperative RT, whereas postoperative RT is usually reserved for close or positive margins after surgery since local recurrence is very common in this setting. Late recurrences may occur within the postoperative period, thus close follow-up of at least 5 years is recommended. Microscopically, spindle-shaped fibroblasts surrounded by rich fibrous stroma lacking mitotic figures are the prominent histopathological features of DFSP (Figure 1). DFSP commonly arises in hairy skin, face and neck, however it may also be seen in different localizations throughout the body. Insidious tumor growth may preclude prompt detection and diagnosis of DFSP. CD34 (Figure 2) and apolipoprotein-D are highly expressed in DFSP with ambiguous

prognostic significance [5]. Differential diagnosis of DFSP includes epidermal inclusion cyst, keloid, hypertrophic scar, malignant melanoma and metastatic carcinoma [6]. The incidence of DFSP is increasing in women and decreasing in men albeit with male predominance. Typical disease presentation is at the fourth decade. Although histologically low-grade and borderline, DFSP has a high propensity for local recurrence after simple excision. Fibrosarcomatous changes and multiple recurrences may be seen throughout the life span of DFSP patients. To minimize the risk of local and distant failure, optimal tumor control should be achieved with rigorous treatment notwithstanding cosmetic considerations. In the management of DFSP, excellent outcomes may be achieved with a multidisciplinary treatment approach [7]. Surgery is a *sine-qua-non* for cure, and resection with wide clear surgical margins provide high local control rates of over 90%. Mohs micrographic surgery, an emerging surgical technique, provides superior local control rates compared to wide local excision [8,9]. However, complete surgical removal of the tumor may not be achieved in some circumstances and development of local recurrence remains a major problem in the setting of positive or close surgical margins. Definitive RT is a viable therapeutic option in the management of patients with unresectable tumors or in the presence of positive surgical margins [10]. Distant metastasis develops in 5% of DFSP patients despite surgery. Imatinib, a molecular-targeted agent, is currently used as another therapeutic option in the management of patients with unresectable, metastatic DFSP [11]. In this study, we evaluated the role of RT in the management of DFSP, making also a literature review.

Methods

Twenty-eight patients with DFSP, treated at GMMMA, Department of Radiation Oncology, between 1974 and 2012 were studied. Parameters including age, gender, surgical method, RT dose, DFS, tumor localization and lesion size were assessed. Twenty-five out of 28 patients (89%) received postoperative RT and 3 patients received definitive RT alone. The 3 patients treated with definitive RT were not amenable to surgery due to critical tumor localization and comor-

Table 1. Patient, disease and radiotherapy characteristics

Patient number	Age (years)	Gender M/F	Location	Stage	RT dose (Gy)	Follow-up (months)
1	88	M	H/N	IA	60	120
2	43	F	T	IA	60	60
3	30	F	E.	IB	66	60
4	21	M	T	IB	66	120
5	28	M	E	IB	60	60
6	30	M	E	IA	70	120
7	29	M	E	IA	60	120
8	22	F	T	IA	60	60
9	23	M	E	IB	60	60
10	20	F	E	IA	66	24
11	26	M	E	IB	60	24
12	35	M	E	IB	60	60
13	23	M	E	IA	60	60
14	36	M	H/N	IB	60	120
15	20	F	T	IB	66	60
16	25	F	E	IA	60	60
17	26	M	E	IB	66	24
18	21	M	T	IA	60	24
19	38	M	H/N	IA	70	60
20	24	F	E	IA	66	120
21	27	F	E	IA	60	60
22	29	M	E	IA	66	120
23	22	M	E	IA	60	120
24	21	M	T	IA	70	60
25	24	M	H/N	IA	60	120
26	27	M	E	IA	66	120
27	31	F	E	IA	66	120
28	23	F	E	IA	66	120

M: male, F: female, H/N: head/neck, E: extremity, T: trunk

bidities. Eleven patients in the postoperative RT group were delivered RT because of recurrent disease after surgery and the other 14 patients were irradiated owing to postoperative surgical margin positivity. In the postoperative group of 25 patients, the types of surgical excision were limited excision in 5 patients and wide excision in 20 patients. Three patients treated with definitive RT were diagnosed by incisional biopsy only.

Radiotherapy

Median RT dose was 63.21 ± 3.7 Gy (range 50-70) in 25-35 fractions. RT was delivered using a Linear Accelerator (LINAC) (Elekta Synergy, UK) or Co-

60 Teletherapy Device at GMMA Radiation Oncology Department. Co-60 was used between 1974 and 1996, and from 1996 till 2012 LINAC-based therapies were introduced. Scar tissue was marked with flexible wire during RT simulation. Intravenous contrast was used in the simulation process to improve tumor localization. The planning computed tomography (CT) images with 2.5 mm slice spacing were acquired using CT-simulator (GE Lightspeed RT, GE Healthcare, Chalfont St. Giles, UK) and were sent to Sim MD simulation and localization software (Advantage SimMD, GE, UK) for contouring treatment volumes and organs-at-risk. Planning Target Volume

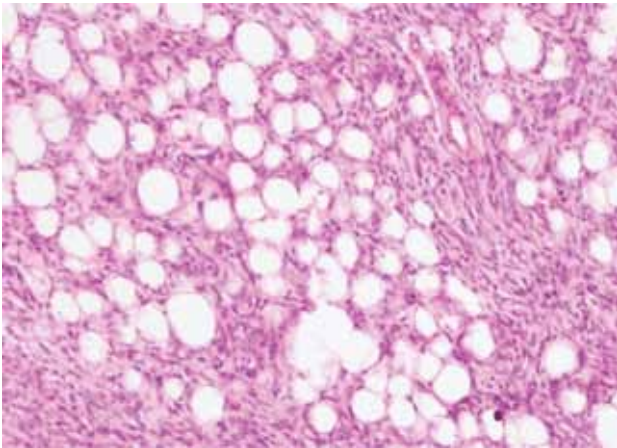


Figure 1. Tumor infiltrating the deeper dermis and subcutaneous adipose tissue. Characteristic honeycomb pattern with neoplastic cell infiltration between adipocytes in adipose tissue (H&E, x40).

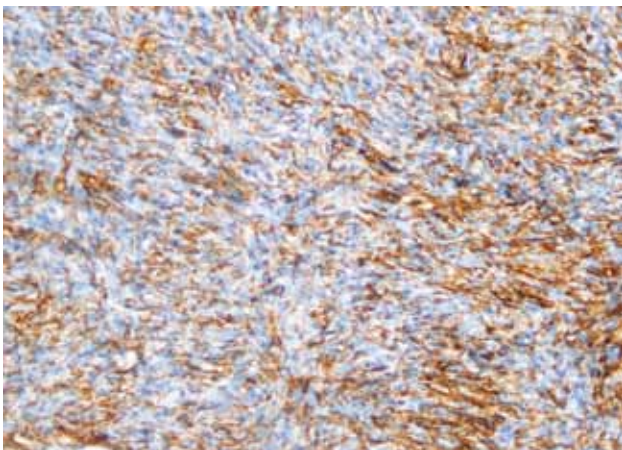


Figure 2. Cytoplasmic and membranous diffuse CD34 positivity (immunohistochemistry, x40).

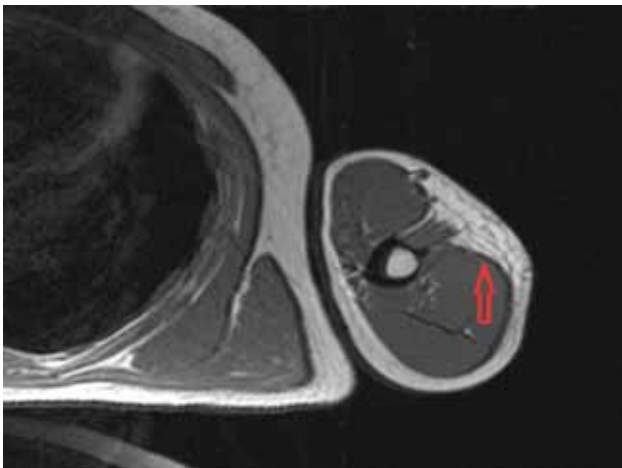


Figure 3a. Preoperative CT of a DFSP patient. Arrow shows the subcutaneous tumor.

(PTV) was generated by adding 2-3 cm mediolateral and 5-8 cm craniocaudal margins to the Clinical Target Volume (CTV). Two cm normal tissue was spared for lymphatic drainage mediolaterally. After treatment planning, RT was delivered under image guidance for set-up verification. Preoperative and posttreatment CT of a patient is shown in Figure 3a and 3b.

Statistics

The Statistical Package for Social Sciences, version 16.0 (SPSS Inc., Chicago, IL) software was used for statistical analyses. Student's-t test was used to assess the relationship between RT dose and survival whereas Fischer's exact test was used to determine the relationship between gender and survival. Five-year DFS of the patients receiving RT after wide excision or limited excision were comparatively evaluated; 5-year OS survival was also determined. The level of significance was set at $p < 0.05$.

Results

Age, gender, surgical method, RT dose, tumor localization and lesion size were evaluated statistically. Patient characteristics are shown in Table 1. Median age was 26 years (range 20-88). Eighteen out of 28 DFSP patients (64.3%) were male and 10 (35.7%) female. The DFSP localization was 64% in the extremities, 22% in the trunk and 14% in the head and neck region. Eighty percent of the surgically treated patients (N=20) had

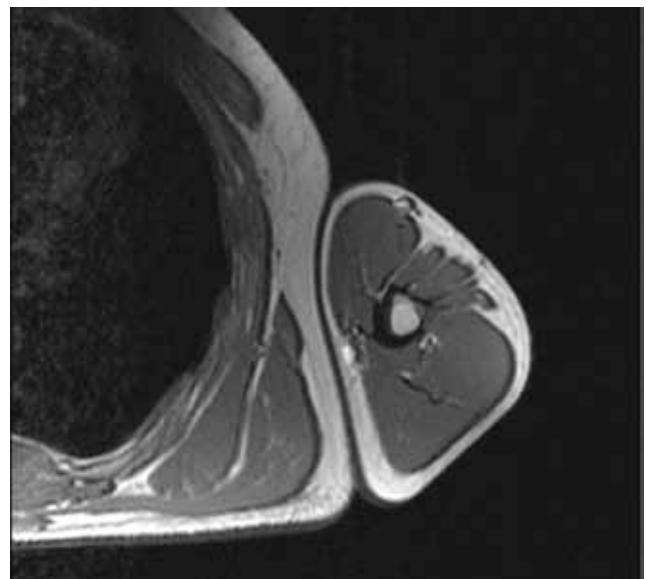


Figure 3b. Posttreatment CT of the DFSP patient in Figure 3a.

excisional biopsy and 20% (N=5) had limited excision. Diagnostic incisional biopsy was performed in 3 patients receiving definitive RT. These 3 patients treated with definitive RT were not amenable to surgery due to tumor localization in close proximity to nerves and vessels or had severe comorbidities precluding surgical resection. The median lesion size according to the examination of the postsurgery or biopsy pathological material was 5.2 cm (range 2.1-8.4). The median RT dose delivered was 63.21 ± 3.7 Gy (range 50-70). The RT doses varied according to postoperative or definitive treatment intent along with improvements in the planning systems in years. All patients were followed up for a median of 80.57 months (range 24-120). The 5-year OS of the whole study group was 93%. Local recurrence occurred in 3 of the 5 locally excised patients with limited surgery and 2 patients with recurrence died of pulmonary metastases. Student's-t test was used to assess the relationship between RT dose and OS, revealing no statistically significant effect of RT dose on OS ($p > 0.05$). Fischer's exact test was used to determine the relationship between gender and survival and showed statistical significance in favor of women compared to men ($p < 0.05$). Five-year RFS of the 20 patients receiving RT after wide excision was 89.6% and 5-year RFS of the 5 patients who were treated with adjuvant RT after limited excision was 74%, revealing statistically significant difference between limited excision+RT vs. wide excision+RT groups ($p < 0.05$).

Discussion

DFSP is a highly recurring but rarely metastasizing cutaneous low-grade malignancy. DFSP is genetically related with Col1A1 gene in chromosome 17 and PDGFB (platelet derived growth factor B) in chromosome 22 [12]. Surgery is the cornerstone of treatment and definitive surgery may be performed as wide excision or as Mohs micrographic surgery [13,14]. In our study, 25 out of 28 patients underwent definitive surgery, however, Mohs micrographic surgery was not used in any patient. Systemic chemotherapeutic agents were used for many years in high-grade DFSP with limited success, whereas molecular-targeted therapy agents are expected to introduce encouraging results as the molecular biology of the disease be-

comes more clearly understood. During follow-up, 2 out of 28 patients had local recurrences and underwent repeated limited excisions. These 2 patients died of distant failure and none of them received imatinib. In case of complete resection but with close surgical margins, some authors favor no adjuvant RT, considering the chance of reexcision in a future recurrence, surgical margin positivity, large tumor size along with unresectable tumor sites requiring postoperative RT with minimal morbidity. However, optimal tumor control is required to minimize the risk of local and distant failure, and incorporating RT to the management of these patients with close surgical margins may improve treatment outcomes. Clearly, recent technological advances have substantially improved the toxicity profile of radiation delivery, thus making RT a viable therapeutic option. Dose-response relationship is not clear for postoperative RT and doses of 50-55 Gy are used for either subclinical or gross disease. Achieving tumor response may take 6 months, but it may also take longer, about 1-2 years in the course of follow-up [15]. DFSP is a rare soft tissue tumor. It is locally aggressive and has a high propensity for recurrence after excision. In a study by Marks et al. with 10 patients, 3 DFSP patients received definitive RT of 66.7-75 Gy and 7 of them received adjuvant RT at a dose of 60-67 Gy to eradicate microscopic residual disease [16].

The definitive and postoperative RT doses we used in our study are consistent with the literature. In a study by Dagan et al., 10 DFSP patients were delivered postoperative RT and 9 of them had a significantly long DFS after the completion of therapy. One patient out of 10 had recurred early and died of the disease. No acute or late complications were noted [17]. In another study by Mendenhall et al., local control rate was over 85% with postoperative RT in cases with surgical margin positivity or close surgical margins. The experience in usage of RT was evaluated in unresectable macroscopic DFSP patients [4]. DFSP is a moderately radioresponsive tumor. Ballo et al. recommended adjuvant RT doses of 50-60 Gy in case of postoperative surgical margin positivity [2]. Ni et al. reported 28 DFSP patients in the head and neck region; local recurrence significantly decreased with wide excision and adjuvant RT essential in

the setting of close surgical margins and surgical margin positivity recommending immediate reconstruction for large defects [18]. Recently, imatinib, a tyrosine kinase inhibitor, is used as an effective treatment option [19].

RT can be used alone or after surgery in cases with positive surgical margins and can increase local control and DFS rates. Multidisciplinary approach may increase local control rates in the management of DFSP [20,21]. Studies on the role of RT in the treatment of DFSP are limited and this study is upholding the use of adjuvant and definitive RT in the management of DFSP where surgery is a *sine-qua-non* for cure. In conclusion, DFSP is a rare disease benefiting from the combination of conservative surgery with adjuvant RT, resulting in high local control rates. This retrospective study with a limited number of patients suggests that RT is an effective treatment modality in DFSP management that can be used after surgery in cases with positive surgical margins, postoperative recurrences and as the sole definitive therapy for patients with unresectable disease.

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Administration of contrast media just before cisplatin-based chemotherapy increases cisplatin-induced nephrotoxicity

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Summary

Purpose: There is a clinical need to predict the probability of cisplatin-induced nephrotoxicity (CIN) in order to make decisions about patient management and relevant preventive measures. The purpose of this study was to develop a risk prediction methodology of CIN.

Methods: 197 consecutive cancer patients, whose serum creatinine was measured at least 48 h before every cycle of cisplatin-based chemotherapy, were included in the study. Demographic and clinical data were collected from the patient medical records. Renal function was evaluated at least 48 h before treatment (day 0) of each cycle, based on the Modification of Diet in Renal Disease (MDRD) formula. CIN was defined as a decrease of $\geq 25\%$ in glomerular filtration rate (GFR) compared to baseline GFR values.

Results: The mean age of the study population was 54.5 ± 9.6 years. Fifty-eight patients (29.4%) whose GFR had decreased by at least 25% compared to baseline values formed the CIN group, and the remaining 139 patients formed the non-CIN group. No significant differences were noted between the CIN and non-CIN groups in terms of age, gender, body mass index and smoking history. Metastatic disease was similar in both groups ($p=0.86$). History of hypertension ($p=0.81$), diabetes mellitus ($p=0.72$), and cardiovascular disease ($p=0.58$) were similar in the two groups. Chemotherapeutic agents used concurrently with cisplatin were similar in both groups. Significantly more radiologic examinations using contrast media were performed in the CIN group compared with the non-CIN group ($p=0.01$). In patients exposed to contrast media within a week before cisplatin administration, the risk of CIN was 2.56-fold higher (95% CI 1.28-5.11) than in patients without such exposure ($p=0.009$).

Conclusion: In patients with exposure to contrast media within a week before cisplatin administration, the risk of CIN was significantly higher than in patients without such an exposure. No additional risk factors for CIN were found in this retrospective observational study.

Key words: chemotherapy, cisplatin, contrast media, nephrotoxicity

Introduction

Cisplatin is a potent and an effective chemotherapeutic agent used to treat various types of cancers including sarcomas, some carcinomas (e.g. small cell lung cancer, ovarian cancer), lymphomas, and germ cell tumors [1]. The most important dose-limiting adverse effect of cisplatin is renal tubular dysfunction manifested as a decline in the GFR and a cumulative impairment in renal function [2]. Nephrotoxicity with cisplatin-based chemotherapy regimens had been observed in more than 50% of the cases in some of the early trials prior to the use of intensive hydration regimens [2]. Despite aggressive hydration, which is routinely applied in the clinic, renal dysfunction still continues to occur [3]. Therefore, several attempts have been made to reduce nephrotoxicity, either by hydration and forced diuresis (administration of aggressive hydration, mannitol, furosemide) or by an alternate method of administration of cisplatin. Risk factors for cisplatin nephrotoxicity include high peak plasma free platinum concentrations, previous exposure to cisplatin, preexisting kidney damage, and the concomitant use of other nephrotoxic agents [4,5]. In an analysis of 400 patients investigating risk factors of nephrotoxicity De Jongh et al. reported that older age, smoking, female gender, hypoalbuminemia, and paclitaxel co-administration are potentially associated with CIN [6,7].

The incidence and severity of renal toxicity increases with repeated usage of cisplatin-based chemotherapy and CIN can consequently become irreversible. As a result, cisplatin discontinuation is generally indicated in those patients who show evidence of progressive renal impairment. There is a clinical need to predict the probability of CIN in order to make decisions about patients' management and take preventive measures. The purpose of the present study was to develop a risk prediction methodology of CIN due to lack of clear results from previous studies.

Methods

One hundred and ninety-seven consecutive cancer patients treated with cisplatin-based combination chemotherapy in the Department of Medical Oncology of the Ankara Numune Training and Research

Hospital between January 2007 and December 2010 were included in this study. These patients had serum creatinine measured at least 48 h before each cycle of cisplatin-based chemotherapy. Demographic and clinical data including age, performance status, tumor characteristics and co-morbid diseases were collected from medical records. Patient performance status (PS) was evaluated by using the Eastern Cooperative Oncology Group (ECOG) scale. Toxicity evaluation was conducted according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0. Cisplatin was administered once every 3 weeks at doses of 75-80 mg/m² at a 20-40 mg/m²/h infusion rate. Cisplatin was administered concurrently with either vinorelbine, paclitaxel, etoposide or gemcitabine. In patients with contrast-enhanced examinations omnipaque 100 ml (osmolarity 0.64 OSM/kg H₂O at 37°C), a non ionic low-osmolarity contrast medium was used. Renal function was evaluated at least 48 h before treatment (day 0) of each cycle; GFR was calculated based on the MDRD formula which estimates 4 variables: serum creatinine, age, race and gender: $186 \times (\text{serum creatinine [mg/L}^{-1.154}] \times (\text{age [years]})^{-0.203} \times (0.742 \text{ if female}) \times (1.210 \text{ if of African descent})$. CIN was defined as a decrease of at least 25% in GFR compared to baseline GFR values. Patients with baseline GFR < 60 ml/min were excluded from the study.

Hydration protocol of cisplatin-based chemotherapy

Intravenous infusion of 500 ml saline in 60 min was followed by infusion of gemcitabine, paclitaxel, vinorelbine or etoposide in another 500 ml of saline. Then, cisplatin infusion with 1000 ml saline was given in 4 h, followed by another 500 ml saline (at least 2500 ml saline in total). Intravenous magnesium sulfate 3 g were administered as standard treatment to every patient. All patients were administered antiemetics.

Statistics

Statistical analyses were performed by using SPSS for Windows version 18.0 (SPSS, Chicago, IL). Patients were divided into two groups according to whether they developed nephrotoxicity. Baseline characteristics of patients who developed CIN were compared

Table 1. Baseline characteristics by cisplatin nephrotoxicity

Characteristics	Cisplatin nephrotoxicity		p-value
	Yes N (%)	No N (%)	
Total	58 (100)	139 (100)	
Age (years)			
<50	18 (31.0)	44 (31.6)	0.87
≥50	40 (69.0)	95 (68.4)	
Sex			
Female	8 (16.0)	19 (13.6)	0.57
Male	50 (84.0)	120 (86.4)	
Body mass index (kg/m ²)			
< 30	24 (41.3)	54 (38.8)	0.47
≥ 30	34 (58.7)	85 (62.2)	
Smoking			
No	11 (19.0)	25 (17.9)	0.88
Yes	39 (67.2)	91 (65.5)	
Ex-smoker	8 (13.8)	23 (16.6)	
ECOG PS			
0	3 (5.1)	11 (7.9)	0.52
1	39 (67.2)	99 (71.2)	
≥ 2	16 (27.7)	29 (20.9)	
History of NSAID usage			
No	40 (69.0)	99 (71.2)	0.23
Yes	18 (31.0)	40 (28.8)	
History of bisphosphonate usage			
No	56 (96.6)	135 (97.1)	0.83
Yes	2 (3.4)	4 (2.9)	
Hypertension			
No	52 (89.7)	121 (87.0)	0.81
Yes	6 (10.3)	18 (13.0)	
Diabetes mellitus			
No	53 (91.4)	123 (88.5)	0.72
Yes	5 (8.6)	16 (11.5)	
Congestive heart failure			
No	56 (96.6)	136 (97.9)	0.58
Yes	2 (3.4)	3 (2.1)	
Type of cancer			
Lung	40 (69.0)	98 (70.5)	0.35
Pancreas	16 (27.7)	38 (27.4)	
Stomach	2 (3.4)	3 (2.1)	
Metastasis at presentation			
No	15 (25.9)	39 (28.1)	0.86
Yes	43 (74.1)	100 (71.9)	
Cisplatin combination with			
Gemcitabine	28 (48.3)	68 (48.9)	
Navelbine	6 (10.3)	10 (7.2)	0.79
Taxanes	5 (8.6)	13 (9.4)	
Etoposide	19 (32.8)	48 (34.5)	
Anemia			
No	44 (75.9)	110 (79.2)	0.46
Yes	14 (24.1)	29 (20.8)	
Hypokalemia (serum K < 3.5 mEq/L)			
No	3 (5.2)	5 (3.6)	0.55
Yes	55 (94.8)	134 (96.4)	
Hypoalbuminemia (serum albumin < 3.5 g/dl)			
No	8 (13.8)	22 (15.8)	0.11
Yes	50 (86.2)	117 (84.2)	
History of administration of contrast media			
No	15 (25.9)	61 (43.8)	0.01
Yes	43 (74.1)	78 (56.2)	

Table 2. Baseline characteristics according to contrast exposure

Characteristics	Contrast exposure		p-value
	Yes N (%)	No N (%)	
Total	121 (100)	76 (100)	
Sex			
Female	18 (14.9)	9 (11.8)	0.35
Male	103 (85.1)	67 (88.6)	
History of NSAID usage			
No	86 (71.1)	53 (69.7)	0.11
Yes	35 (28.9)	23 (30.3)	
History of bisphosphonate usage			
No	118 (97.5)	73 (96.1)	0.42
Yes	3 (2.5)	3 (3.9)	
Hypertension			
No	106 (87.6)	67 (88.2)	0.54
Yes	15 (12.4)	9 (11.8)	
Diabetes mellitus			
No	107 (88.4)	68 (89.5)	0.50
Yes	14 (11.6)	8 (10.5)	
Congestive heart failure			
No	120 (99.2)	73 (96.1)	0.16
Yes	1 (0.8)	3 (3.9)	
Type of cancer			
Lung	80 (66.1)	50 (65.8)	0.24
Pancreas	38 (31.4)	24 (31.5)	
Stomach	3 (2.5)	2 (2.6)	
Metastasis at presentation			
No	83 (68.6)	50 (65.8)	0.17
Yes	38 (31.4)	26 (34.2)	
Chemotherapy combination with			
Gemcitabine	58 (47.9)	35 (46.1)	0.21
Navelbine	11 (9.1)	5 (6.5)	
Taxanes	10 (8.3)	11 (14.5)	
Etoposide	42 (34.7)	25 (32.9)	

with non-CIN group with χ^2 test (for categorical variables) or two-sample T-test (for continuous variables). Logistic regression analysis was used in order to test baseline parameters for their prognostic value regarding toxicity. Log-rank test was used to examine the statistical significance of the differences observed between the groups. Two-sided p-values of <0.05 were considered statistically significant.

Results

The mean age of 197 patients was 54.5 ± 9.6 years. Fifty-eight patients (29.4%) whose GFR decreased by at least 25% compared to baseline GFR were included

in the CIN group, and the remaining 139 patients were included in the non-CIN group. Cisplatin-based chemotherapy was used mostly in patients with non-small cell lung cancer (N=98), pancreatic cancer (N=54) and small cell lung cancer (N=40) in this study population. Distribution of the disease types did not differ significantly between the CIN and non-CIN groups. Baseline demographic and clinical characteristics are listed in Table 1. There was no difference in terms of age, gender, body mass index and smoking history between the two groups. Baseline mean serum creatinine levels were 0.84 ± 0.2 and 0.88 ± 0.2 in the CIN and non-CIN groups, respectively ($p=0.12$).

The baseline GFR values as calculated by the MDRD formula were 98 ± 26 ml/min in the CIN group and 94 ± 24 ml/min in the non-CIN group ($p=0.22$). The mean serum albumin levels were 4.0 ± 0.6 g/dl in the CIN group and 4.1 ± 0.7 g/dl in the non-CIN group ($p=0.11$). The mean serum uric acid and potassium levels were similar between the two groups.

Metastatic rate was similar in both groups at the time of diagnosis ($p=0.86$). Also, history of hypertension ($p=0.81$), diabetes mellitus ($p=0.72$), and cardiovascular disease ($p=0.58$) were similar in the two groups. Chemotherapeutic agents used concurrently with the cisplatin chemotherapy were similar in the CIN and non-CIN groups (Table 1). Median cisplatin cycles were 4 (range 1-12) in both groups. Mean total cisplatin dose was 325 ± 143 mg/m² and 362 ± 180 mg/m² in CIN and non-CIN groups, respectively ($p=0.30$). For response evaluation 121 patients (61.4%) had chest and abdominal CT scan. The mean age of the patients with contrast exposure was 54.5 ± 0.9 years, whereas it was 54.4 ± 1.0 years in the non-contrast exposure patients ($p=0.91$). The pre-contrast exposure GFR value was 101 ± 2.6 ml/min, whereas the post-contrast exposure GFR was 86.5 ± 3.2 ml/min ($p=0.01$). Radiologic examinations with contrast media were performed more frequently in the CIN group as compared to the non-CIN group ($p=0.01$). In patients exposed to contrast media within a week before cisplatin administration, the risk of CIN was 2.56 (95% CI 1.28-5.11) fold higher than in patients without exposure to contrast media ($p=0.009$) (Figure 1). CIN was seen in 45.6% of the patients exposed to contrast media within a week before cisplatin administration and in 19.4% of the patients not exposed to contrast media ($p=0.01$). Two patients in the CIN group required hemodialysis and both these patients were exposed to contrast media within a week before the administration of cisplatin.

Discussion

In the present study, nephrotoxicity developed in 29.4% of the 197 patients treated with cisplatin-based chemotherapy. In both groups, a median of 4 cycles of cisplatin-based chemotherapy was given every 3 weeks with a median cisplatin dose of 320 mg/m².

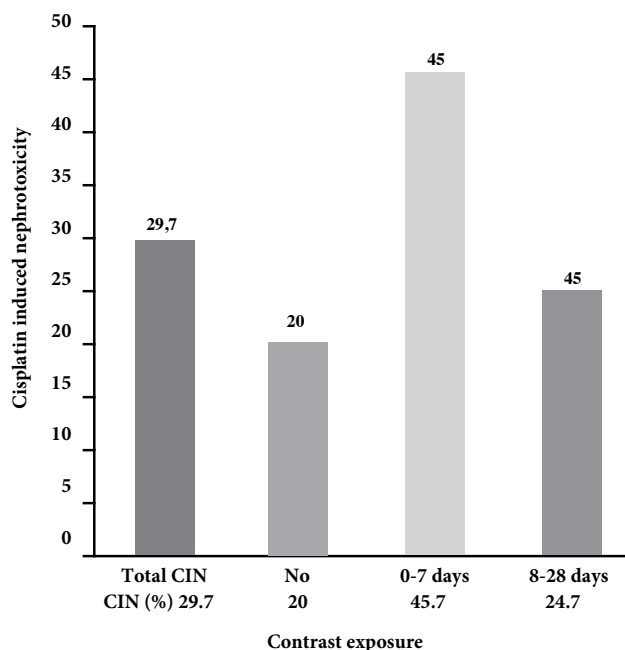


Figure 1. Rate of cisplatin-induced nephrotoxicity (CIN) according to contrast exposure (p value is 0.01 between no contrast exposure and contrast exposure between 0-7 days; p value is 0.02 between contrast exposure 0-7 days and 8-28 days).

Chemotherapeutic agents administered concurrently with cisplatin were similar in the CIN and non-CIN groups. Previously described potential risk factors for CIN such as hypoalbuminemia, hypokalemia, smoking history, gender, and older age, were also investigated in this study, but no relationship with nephrotoxicity was found with any of the aforementioned parameters. This retrospective observational study showed that exposure to contrast media within a week before administration of cisplatin increased the risk of CIN by 2.56 fold. To our knowledge, this study is the first to show the relationship between CIN and cisplatin-based chemotherapy.

Cisplatin nephrotoxicity generally manifests as a reduction in GFR due to renal tubular dysfunction and the risk of nephrotoxicity increases with repeated cycles of chemotherapy [8]. Several mechanisms are responsible for renal dysfunction following cisplatin administration. These mechanisms are tubular epithelial cell toxicity, vasoconstriction in the renal microvasculature and pro-inflammatory effects of cisplatin [9]. More than 50% of the drug is excreted in the

urine in the first 24 h following cisplatin administration and the concentration of platinum achieved in the renal cortex is several fold greater than in plasma and other organs [9]. Because of its low molecular weight, cisplatin is freely filtered in the glomerulus [10]. Cisplatin primarily injures the S3 segment of the proximal tubule, causing a decrease in the GFR [11]. Cisplatin can also cause vasoconstriction in the renal microvasculature, thus leading to decreased renal blood flow [12]. Contrast media can precipitate CIN. Renal vasoconstriction is a common finding of contrast nephropathy which is mediated by contrast-induced release of endothelin and adenosine and by the high osmolality of the contrast agent [13,14]. Besides, contrast media can cause tubular injury as a result of direct cytotoxic effects or in association with the generation of oxygen free radicals [15]. Because both cisplatin and contrast media can cause nephrotoxicity by same mechanisms, it is not surprising that CIN develops more frequently in patients exposed to contrast media as seen in our study. Raschilas et al. [16] reported severe acute renal failure after administration of contrast media in a patient treated with cisplatin. Oymak and colleagues [17] also reported the induction of an irreversible acute renal failure following intraperitoneal cisplatin chemotherapy and contrast media injection in a woman treated for ovarian cancer.

Despite aggressive hydration with saline, which is routinely applied in the clinic like in our study, nephrotoxicity still occurs. Therefore, several preventive attempts should be done to reduce CIN. Mannitol is frequently used to induce diuresis; however there is no convincing data that mannitol and other diuretics may attenuate cisplatin nephrotoxicity [18]. N-acetylcysteine, a thiol derivative, may have some role in preventing cisplatin nephrotoxicity in high risk patients, but there are contradictory results in the prevention of contrast nephropathy [19,20]. Benoehr et al. [21] reported that theophylline may prevent CIN in a small clinical trial.

Due to the high incidence of CIN with the currently used platinum compounds, new less nephrotoxic platinum formulations have been investigated. Jehn et al. [22] reported that liposomal formulation of cis-

platin (lipoplatin) reduces renal toxicity compared to conventional cisplatin. In another study, no nephrotoxicity was observed in the second-line therapy for small cell lung cancer with a platinum derivative picoplatin [23]. Until less nephrotoxic compounds of platinum derivatives are widely available, it seems suitable to avoid concomitant nephrotoxic agents and volume depletion during conventional cisplatin treatment.

Due to the lack of large, significant studies, optimal therapy to prevent CIN remains unclear. Radiologic procedures, mostly with contrast media, are widely used to evaluate the response to chemotherapy. In conclusion, we suggest that radiographic procedures with intravenous contrast material should be delayed for at least 1 week in patients receiving chemotherapy with cisplatin.

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Structure and function of the oncologic boards in Greece. Description of the institutional and scientific frame; objective problems and difficulties

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Summary

Purpose: Oncology boards should constitute a routine in all hospitals that are dealing with the care of cancer patients. Unfortunately the procedure which should be followed to deal with this health problem has some deficiencies.

Methods: A literature review has recently been attempted, searching Internet databases by using key words such as oncologic board, medical legislation and medical ethics.

Results: Current mentality suggests that hiding the truth from the patient is wrong and unethical. However, in the Greek society, this is not the case as it seems not right to adopt foreign practices, i.e. to disclose directly to the patient all information relevant to his health status, the intended therapy and possible outcome. Instead, ambiguous information pass onto relatives who in turn bear the burden of informing the patient.

Conclusions: The best solution would be the integration of the positive elements of the patient's awareness and the beneficial effects of the involvement of the Greek family in the general care of the cancer patient.

Key words: ethics, oncologic boards, regulatory affairs, social-medical study

Introduction

In Greece, malignancies constitute the second cause of mortality (23%) and the third of morbidity (9.4%) with apparently increasing trends. During their treatment, patients suffering from cancer seem to have a series of ethic and practical dilemmas, intermingled with the way the delivery of health resources is practiced. The function of an oncologic board is imposed for the above mentioned reasons before the application of any kind of treatment. The oncologic board must be composed by surgeons, medical oncologists, radiation oncologists and pathologists.

Methods, Results and Discussion

A literature review has recently been carried out. Internet databases were searched using key words such as oncologic board, medical legislation and medical ethics.

In Greece the institutional framework of an oncologic board is defined by the regulations of Medical Deontology 25/1955(A171), articles 27, 28, 29 and 30 (when and where it is convoked, its powers etc), the Declaration of Amsterdam (briefing, patients' rights), the law 3209 (24 -12-2003, page 5206, paragraph 2, over the formation and operation of the oncologic board of the Hospital) [1], and the Medical Code of Deontology /2005(briefing and patient's acceptance). Briefing is not a simple procedure, especially for those who suffer from cancer and constitute a social stigma. Moreover, the convocation of the board is not accompanied by a written binding deduction.

It is underlined that most of the time participants of an oncologic board discuss about patients without having seen them, while they have to take fundamental decisions about their health. It is thus understandable that quite often therapy has to be changed according to new data. That is to say that social factors and demographic data of each patient have to be taken into consideration.

It is clear that the physician is not legally obliged to heal the patient but to do his best to provide his services assiduously according to the scientific progress made up to that date.

Human life is protected by the Law in any form and under any circumstances. A fatal disease neither

negates nor restricts the staff's obligation to give the patient the proper care.

Generally speaking, physicians and nurses have an increased obligation to take care of patients and this is due not only to the possible danger which threatens human life and health but also to the relation based on the confidence between the patient and the doctor.

Consequently, doctors have to do their job according to the regulations and their knowledge of the technological advances in medicine (*lege artis*), otherwise compensation rights may be asked by the patients if health damage is proved or if doctors or health staff have not fulfilled their duties [2].

Furthermore, it is crucial to point out that patient's rights regarding legal matters and the relationship between physicians and patients are described in Law texts or in Declarations such as the one of Lisbon (Table 1).

According to the present legislation, life is the milestone of our civilization and therefore it is worth protecting it under any circumstances, even if the patient or his relatives think otherwise. According to the article 299 of the Penal Code, whoever takes human life is charged with homicide and he is sentenced to life imprisonment or he is put in jail for 5 to 20 years.

The first contemporary Greek Medical Deontology Code (Greek Government Gazette 171-A-16-7-55) [3] refers to the patients' rights in its articles 8

Table 1. Patients' human rights (Declaration of Lisbon)

The patient has the right to choose his physician freely.
The patient has the right to be cared for by a physician who is free to make clinical and ethical judgments without any outside interference.
The patient has the right to accept or to refuse treatment after receiving adequate information.
The patient has the right to expect that his physician will respect the confidential nature of all his medical and personal details.
The patient has the right to die in dignity.
The patient has the right to receive or to decline spiritual and moral comfort including the help of a minister of an appropriate religion.

and 9. The Penal Code points out that omissions or negligence during daily medical practice are considered to be 'punishable' (articles 300, 301 and 302) [4].

Social instructions are based on this spirit in the Law 2071-92, articles 47, 61, and 62, as far as the patients' rights in hospitals are concerned.

The Law 2519-1997, Greek Government Gazette 165, about Regulations in the National Health System emphasizes in his first article the civilians' rights to benefit from health services. The legislator also recommends the creation of a special committee with specific responsibilities for the protection of patients. A committee will also be set to facilitate the communication between doctors and patients.

The independent management by an advocate of health and social solidarity is established with the Law 3293-2004. This one is incorporated to an independent authority managed by ombudsman who has already provided services to any civilian in need of public health services. His jurisdiction has to do with the rehabilitation and the protection of any civilian and the transmission of the case to the relevant Ministry. The advocate for health and welfare examines the legality of individual administrative acts or omissions which may occur by the Health sector and which is pointed out by affected citizens. His intervention may appear after the civilians have submitted their case to the implicated Health Service. Furthermore, this advocate has the right to mediate in cases which concern the Ministry of Health and Social Solidarity, the regional management, insurance organizations, and pension/health care funds, general or specialized hospitals, psychiatric hospitals, health centers, regional and rural clinics etc.

One essential criterion to characterize a medical act as correct is the compliance with obligations by physicians as far as patients are concerned, according to the Medical Deontology and the respect of human life as it has already been mentioned.

In medicine, a clear distinction is often done between technical errors and errors of judgment.

Both errors can be made either during the period of diagnosis or during the treatment period, which consequently could damage the patient's health or even threaten his life. In addition, other errors can be

identified:

- Unnecessary errors, i.e. the ones doctors or nurses are not responsible for as they have done their best to fulfill their mission.
- Liable errors, i.e. the ones doctors and nurses are responsible for as they have made mistakes by omitting asking for the appropriate medical tests or by not achieving what can be done to relieve patients.

An accident is characterized as being random and unpredictable and as one which can damage the patient while doctors and nurses are not responsible for.

The failure of a medical action is specifically defined according to its result. An unsuccessful medical action has as a consequence to hurt the patient either by the non accomplishment of the therapy or by the existence of side effects regardless of the patient's restoration from his initial health condition.

The civil medical liability is divided into two categories:

- 1) The conventional one, which is the agreement made by a patient and a physician about the services provided by the latter of the two. It's in fact a deal with a work contract if the doctor's services are remunerated for a short or a long period of time and with a project contract if the doctor's services are provided for a specific medical act. As a result, a refund can be asked if the agreement is not respected.
- 2) The tortious one. In this case, the conditions asked for a refund are not only foreseen in the article 914 AK but also in some specific regulations. Here are some of the conditions: a) the irresponsible attitude and the lack of consciousness shown by the physician as defined by the Law and the common sense; b) the lack of knowledge, skills and attention which could have provoked a disastrous result; c) the negligence which could cause death or damage; d) the connection between practice and result or omission of medical service and result.

It's up to the judge to decide whether there is malfeasance or if damage is caused by accident, estimating the facts which occur in each case.

It is sometimes possible the damage caused to the patient to concern only his fortune, for instance when the patient has to spend a lot of money for his medi-

cation etc. The physician's specialty is taken into account too as the expenses may be higher due to this fact.

From the above-mentioned, legal penalties or excessive compensation may constitute a serious handicap to medical science and may not boost the right practice of it. The so called 'defensive medicine' is then put forward, i.e. the doctor - in a effort to protect himself against possible charges for negligence - orders unnecessary medical exams which may produce evidence of his innocence but certainly not promot-

ing the patient's welfare. Finally it is needed to point out that, despite the amendments made in the Medical Law (Law 3418/ 2005) to protect patients, same proved inadequate due to the complexity, and inconsistency of Greek legislation [2].

According to the District European Bureau of World Health Organization the content of the patients' briefing should include:

- The procedures concerning the diagnosis.
- The diagnosis itself.
- The various options of treatment, their advantages

Table 2. Greek Medical Code of Deontology 2005; Patient's briefing

Article 1 (meanings, definitions and applications) paragraph 48:

With the word 'intimate' we mean relatives by blood or marriage, foster parents and foster children, husband and wife, the long term companions, siblings, siblings' long term companions or spouses, commissioners and all those who are under judicial support.

Article 9 (doctor's obligations towards his patient), paragraph 1:

Priority is given to the protection of the patient' s health.

Article 11 (briefing obligations):

1. Physicians' duty is to tell the truth. Patients must be fully informed about their real condition, the application and the results of the suggested medical services, the consequences, the risks and complications of its applications, the alternative options and the rehabilitation time which may be needed. Taking then everything into consideration, patients can make up their mind and decide what the best is for them.
2. Physicians respect people's desire not to be informed. In this case, patients have the right to designate a person of their choice so as to be informed about their condition, the results of the suggested medical acts, the consequences and the possible dangers of them.
3. Special attention must be paid when patients are informed about surgical operations such as transplants, assisted reproduction interferences, gender change or rehabilitation and cosmetic surgeries.
4. If patients have not the ability to consent on a medical act, physicians should inform them as much as it is feasible. Other persons who have the authority to take decisions according to the next article must be informed too.

Article 12 (patient's consent):

1. The physician has not the right to act without prior patient's consent. For the rest of the cases, when a person has not the ability to take any decision, it is for the judge to decide or for the designated person what could be done. In each case, the physician must try to ensure the voluntary participation and cooperation of the patient and especially of the one who can comprehend his condition, the dangers and consequences of the medical intervention.
2. Not only the addition of all the positive elements of the autonomy of other societies but also the beneficial influence of the Greek family will lead to face the problem of patients' briefing suffering from malignancies more effectively according to the Greek mentality and not according to the adoption of other informative ways of other societies.
3. Briefing, informed consent and the respect of the patients' autonomy constitute fundamental ethical issues of the relation between doctor and patient. Patients' autonomy has been characterizes as the most common practice in medical ethics [7]. In hospitals, patients' briefing is not an easy matter, especially for the ones who suffer from a fatal disease or connected with a social stigma. Hiding a painful truth is common practice. A clear change has only been noticed in Western societies the last 30 years [8, 9]. The reason of hiding the truth is to give people hope which is crucial for their psychology [9-11].

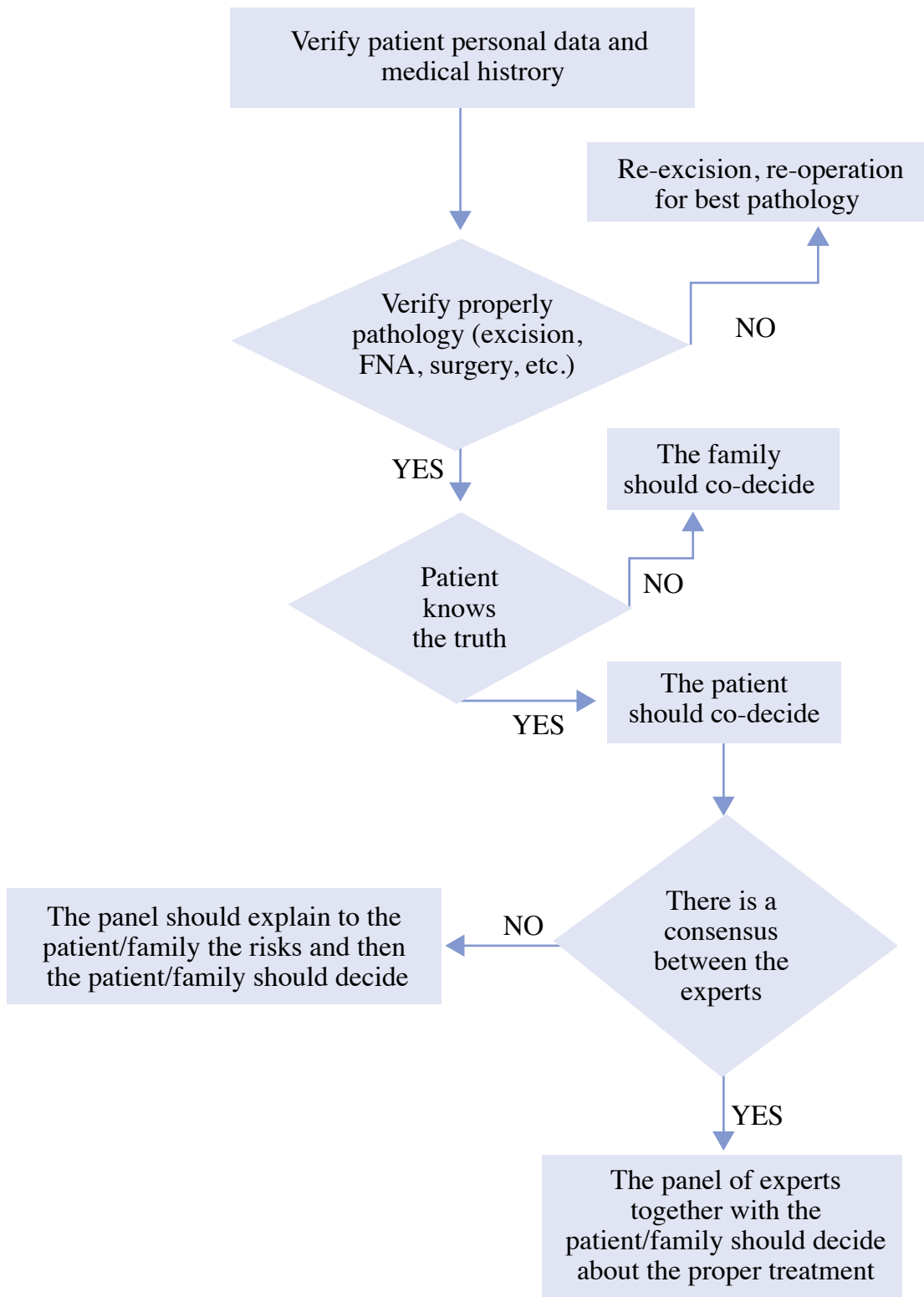


Figure 1. Flow chart with the suggested procedure involving the patient/family.

and disadvantages and their possible consequences.

- The eminent dangers or not of the therapy or the denial of it.
- The procedure of the treatment, its duration and the patients' suffering because of it.
- The prognosis.
- The results and the side-effects of the medication and their interaction with other drugs.
- The status of health and the way of life after treatment.

The Declaration of Amsterdam [5] about patients' rights in Europe in 1994 states that patients should be fully aware of:

- Their health condition and the medical data concerning their disease.
- The suggested medical procedures along with their benefits and drawbacks.
- The alternative options with their results on the diagnosis, the precognition and the course of treatment.

The Code of Medical Deontology, voted in the Greek Parliament, sets rules in our country for the first time which deal with the physician's obligations to inform his patients (Table 2) [6-10].

However, this regulation is not fully compatible to the Greek mentality [11].

In the last 30 years, the way of informing patients has radically changed from a protective concealing to fully revealing the truth condition to the patient. This change results to the human's respect and autonomy and is more intense in North America and North-West Europe. Hiding the truth from cancer patients is still in use in many countries including Greece. A great number of factors contribute to the different policies of information. Kallergis G. reported the methodology by which the information can be disclosed to the patient about his status. The method depends upon the character of each patient [12-15]. The communication among family members should be the determining factor for choosing the appropriate approach for informing them [16, 17].

In societies, such as the Greek one, where the family bonds are still strong, there is a tendency to overprotect sick people from the bad news as the whole family faces the problems and not only one of its members. Consequently, the relation between pa-

tient and doctor is transformed to a relation between family and doctor. This is a Greek reality and it has not been taken into account by the Code of Medical Deontology.

The Code of Medical Deontology which was voted unanimously by the members of the Parliament on 8 November 2005 deals with matters of briefing and patient's consent for the first time. The Code requires the patient's full briefing of his condition apart from the cases where the patient does not choose to be informed or he is not capable of being so [18]. On the other hand, it is well known that the patient is not aware of his condition, particularly after the diagnosis, and only his close relatives are informed. There is then a contradiction between the new Medical Code of Deontology and the traditional practice in our country [19, 20].

In a study made at the 'Aretaieion' University Hospital, only 23% of the patients' relatives suffering from cancer considered that they must be informed of their condition, while 73% of the health staff believed that they should be aware of their disease [21]. Moreover, 89% of the health staff considered that the relatives must be informed too. When health care providers communicate with their patients, they avoid using the word 'cancer' even if they know it and 62 % have difficulties in having a clear conversation with the patients when forecast issues have to be put forward. Forty one percent believed that briefing may lead to the patient's disappointment and isolation.

On the other hand, 71% of the health care providers were convinced that a basic element for the healing process is revealing the truth. Finally, most of the health staff considered telling the truth is the doctor's responsibility [22, 23]. A review of related studies from 1971 to 1987 showed that Greek doctors insist on hiding the diagnosis from their patients and hardly speak of their forthcoming death. On the contrary, more and more patients demanded a full briefing [24]. In a recent Greek poll among 1500 doctors in oncology or general hospitals, 22% revealed the truth and 76% preferred to inform the patient relatives. It is obvious that things tend to change step by step [19, 25, 26].

The change in the briefing procedure is related

with the change of social structures. This change of attitude has begun in the Western societies and demands the person's respect and autonomy even when it comes to medical decisions [27]. This attitude results in a change of series of social-financial character, such as the dense urbanization, the consumers' movement and the criminalization of the medical profession which is reinforced by the involvement of insurance companies. Progress made in healing cancer and therefore a decrease of the fear of diagnosis may be convincing factors of this change of attitude in briefing [28]. Finally, another essential factor seems to be the alteration of the family from the extended traditional type to a more nuclear one.

Conclusions

Oncology boards should be part of the routine function in all hospitals treating cancer patients. Unfortunately the procedure which should be followed to deal with this health problem has some deficiencies.

The Greek Ministry of Health and Social Solidarity (Ministerial decree 141758/12.11.2010) for the structure of the cancer centers dealing with cancer diagnosis and treatment, refers also to submitted proposals with respect to the restructure of oncologic boards [29].

Furthermore, the above amendments make reference to the control / assessment and records keeping in the oncology departments.

In addition, the responsibility of the function the oncology department is given to the Hospital's oncology committee, aiming to provide better services [29].

As far as cancer patients are concerned, the physician is obliged to conform to the patients' rights according to the directive of the European Union, the Hague Declaration and the article 47 of the Greek Law 2071/92 [30].

With the current mentality, hiding the truth from the patient is wrong and unethical. However, in the Greek society this is not the case as it seems not right to adopt foreign practices. On one hand, informing relatives is ambiguous but on the other hand the continuation of this informational policy is wrong.

So the best solution would be the integration of

the positive elements of the patient's awareness and the beneficial effects of the involvement of the Greek family upon one of its members. Thus, the best process for an oncological council should be a flow chart with the alternatives of one or more treatment options, whereas the main aspect should be the inclusion of the patient himself in the procedure of treatment decision. In other words the patient should be aware of his treatment effectiveness as well as of its toxic potential, and the oncological board should co-decide with the patient for the treatment options. This is in accordance with the good medical practice [31], aiming also to the patients' consent, which, no doubt, will lead to the reduction of malpractice (Figure 1) [21]. Further research on the impact of patient decision would improve the structure and the functionality of oncologic boards. In the future, the research should focus on the development of certain guidelines for the integration of expert's opinion and patient's decision.

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LETTERS TO THE EDITOR

Triple negative endometrial cancer may be more sensitive to platinum based chemotherapy

Dear Editor,

Endometrial cancer is the most common gynecologic cancer in the developed world. In 2011, the mortality rate was 1.7-2.4 per 100,00 women. In the United States, as with other developed countries, 46,470 new cases and 8,120 deaths were due to this disease in 2011 [1,2]. Endometrial cancer is divided into type I and II subtypes. Type I is associated with a hyperestrogenic state and usually occurs in obese women, tend to be well differentiated and is identified in early stages. Type II, which is not associated with hyperestrogenism, is usually seen in thin women and is diagnosed in advanced stages. Most endometrial cancers are low-grade, early-stage and carry an excellent prognosis. On the other hand, the 5-year survival of advanced stages (stage 3 and 4) ranges between 23 and 67%.

Breast cancer is also one of the most frequent malignancies in women. The lifetime risk for breast cancer in the United States is usually about 1 in 8 (12%) of women by the age of 95, with 1 in 35 (3%) chance of dying from breast cancer [3]. Breast cancer can be divided in subgroups based on immunohistochemical staining. The group that lacks estrogen receptor (ER) and progesterone receptor (PR) expression and shows absence of HER2 protein overexpression is known as the triple negative phenotype (TNP). TNP breast cancers are more aggressive, have a high proliferation rate, high nuclear grade, frequent p53 overexpression, are more likely to show distant metastasis, and have poorer outcomes, including shorter disease-free and overall survival [4]. TNP breast cancer (TNBC) cells exhibit an abundance of DNA aberrations, suggesting that their DNA repair mechanisms are defective. Consequently, these tumors are theorized of having an increased sensitivity to agents that cause interstrand DNA breaks (e.g., platinum agents). As such, the sensitivity of TNP breast cancers to platinum-based chemotherapy has been the focus of several recent clinical

trials in the neoadjuvant/adjuvant and advanced disease settings. A study conducted by Sirohi et al. has retrospectively evaluated the efficacy of platinum-based drugs in 143 metastatic breast cancer patients. Among these, 93 (63.7%) were TNP. The objective response rate was 33.3% in the TNBC group vs. 22% in the non-TNBC ($p=0.1$) although no difference in OS, PFS and response duration was observed [5]. To summarize, as platinum-based treatments are effective in TNBC, we expect them to be also effective in TNP endometrial cancer. Larger studies conducted in such TNP populations to search effectiveness of platinum-based chemotherapy are needed.

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High dose chemotherapy with autologous stem cell transplantation for patients with germ-cell cancer

Dear Editor,

Germ cell tumors (GCTs) practically represent the only kind of solid tumors that are curable by chemotherapy even when metastatic. This is largely due to their chemosensitivity. Although most of these tumors are cured with the first – or - second-line chemotherapy, patients who experience more than two relapses, or are refractory to all therapy lines, usually die from this disease. The bad prognosis of these patients had urged researchers to search for other treatment options, and having in mind the chemosensitivity of GCTs, a logical step was to increase the doses of cytotoxic drugs and try to overcome the resistance with high-dose therapy [1]. In a retrospective study published in 2008 Einhorn et al. stressed that high-dose protocols for metastatic GCTs can cure a certain number of patients even after multiple relapses [2]. After the publication of this study, our Institute also introduced this procedure using a modified TAXIF protocol of the Tenon hospital, Paris [3,4]. The final confirmation of the activity of high-dose chemotherapy was the last year's retrospective study by Lorch et al. [5] where in 1984 patients they proved that in relapsed GCTs high-dose therapy, followed by autologous hematopoietic stem cell transplantation leads to longer survival when compared to conventional chemotherapy.

During 2008-2010 we performed 11 transplantations in 8 heavily pretreated patients who were previously administered a median of 4 (range 3-4) chemotherapy lines. We used a modified TAXIF protocol, which includes mobilization of stem cells by administering the submyeloablative protocol paclitaxel 250 mg/m² and epirubicin 100 mg/m² with G-CSF 10 mcg/kg. After mobilization, a large-volume apheresis was performed until the harvested number of cells was enough for two transplantations. The high-dose protocol was the combination of carboplatin AUC 4

daily for 5 days plus etoposide 300mg/m²/day, days 1-5. The median number of the administered stem cells per transplantation was 3.6x10⁹ (range 1.9-4.5) CD34+ cells per kg of body weight. The median time for neutrophils engraftment was 10 days, and for thrombocytes 13 days. There were no therapy-related deaths. The most frequent grade 3 / 4 toxicities were febrile neutropenia (100% of patients), thrombocytopenia (100%), colitis / diarrhea (63%), nausea (54%) and oral mucositis (45%). One patient developed veno-occlusive liver disease, while 2 patients developed grade 4 infections (facial phlegmon and one septic shock). Overall clinical benefit rate was 62.5% (37.5% PR, 25% SD). No complete remissions were seen. Median survival was 11 months (range 4-20). High-dose chemotherapy with stem cell transplantation represents an option for heavily pretreated patients with GCTs and is feasible at our Institute, which may be one of the reference centers for this kind of treatment in the Balkans. The treatment results in this group of patients is poor, so earlier intensification is mandatory.

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Colon involvement in a chronic lymphocytic leukemia patient

Dear Editor,

Chronic lymphocytic leukemia (CLL) is the most frequently observed type of leukemia in patients older than 50 years. The prognosis of this disease is much better when compared to other leukemia types [1]. Although CLL primarily involves the bone marrow, extramedullary tissues may also be affected. In CLL patients, gastrointestinal involvement is usually seen in Richter syndrome, which is the disease transformation into large B cell lymphoma [2]. The prognosis worsens in such cases.

A 61-year-old woman presented to our outpatient department with stomach ache in the midline. Physical examination revealed no lymphadenopathy or hepatosplenomegaly. Lab tests revealed white blood cell count 25890 mm³, lymphocyte count 18290 mm³, hemoglobin level 14.2 g/dl, platelet count 208000 mm³, lactate dehydrogenase level 454 IU/dl, while peripheral blood smear demonstrated 72% mature lymphocytes with occasional basket cells. Bone marrow aspiration and biopsy were performed and were consistent with CLL (60% B lymphocytes, CD 20 (+), CD 79 (+), CD5 (+)). A flow cytometry performed showed CD5 90% CD23 80%, indicating that the sample comprised of leukemic cells. Abdominal CT scan revealed thickening of the wall of the transverse colon, after which a colonoscopy was performed. In colonoscopy, the transverse colon mucosa was, in general, minimally edematous, granular and in the rectum there were numerous apparent Peyer plaques. Biopsies were obtained from the rectum and

transverse colon which were consistent with chronic lymphocytic colitis. Microscopically, dense mucosal and submucosal lymphocytic infiltrates were detected in the transverse colon, and 4/7 biopsies of the colon, showed predominance of small CD20 positive lymphocytes. The cells stained positively also for CLL-associated antigens CD5, CD23, and CD43, but were negative for mantle cell-associated antigen cyclin D1. The patient was considered stage 0 CLL with colon involvement. Gastrointestinal involvement is not common in CLL. Colon involvement without Richter syndrome has rarely been reported in the literature. Arkila et al. [3] have reported a 69-year-old man with CLL and anemia in whom colonoscopy was macroscopically normal, but the histological specimens revealed lymphocytic leukemia in the ileum and colon. In our patient we also have demonstrated colon involvement without Richter syndrome. CLL may cause upper gastrointestinal haemorrhage by directly infiltrating the gastroesophageal junction or through bleeding from oesophageal varices caused by CLL-associated splenomegaly and portal hypertension. According to one case report [4], protein-losing enteropathy may be found in CLL patients. Reports also mention gastrointestinal CLL manifestations such as infiltration of the intestinal mucosa in the small bowel as well as CLL presenting as colitis. CLL, especially after Richter transformation, can cause signs and symptoms suggestive of chronic inflammatory bowel disease. Other rarely described CLL manifestations include intussusception, even perforation of the colon and can also form a route

for infectious complications. The histopathologic differential diagnosis of common benign lymphatic hyperplasias and various malignant lymphoid disorders of the intestine may be challenging. The diagnostic range for CLL should include CD20+/CD5+ coexpression with CD23+ phenotype, and negative staining pattern for Cyclin D1 to exclude mantle cell lymphoma. Differential diagnosis of indolent CD5 negative B-cell lymphomas include follicular lymphoma, which usually has CD10+ phenotype, whereas mucosa-associated marginal zone lymphoma lacks specific phenotypic markers and its immunophenotypic diagnosis is mainly based on exclusion. Lymphoepithelial lesions and plasmacytic differentiation are suggestive of MALT-lymphoma [5].

In conclusion, also gastrointestinal evaluation should perhaps be part of a complete assessment of the treatment response and remission status in CLL patients in whom the colon was originally involved. In CLL patients with gastrointestinal symptoms, an endoscopic evaluation must be performed, and biopsies must be obtained from suspicious regions. The fact that CLL patients may have gastrointestinal involvement without Richter syndrome must be kept in mind.

A simple technique for vacuum drainage of the peritoneal cavity for the management of anastomotic leaks in patients with gastrointestinal malignancies

Dear Editor,

In cancer patients, any anastomosis along the gastrointestinal tract carries the risk of an anastomotic leak, the incidence of which can be as high as 20%, depending on several and specific local and systematic factors [1]. Following an anastomotic leak, the content of the gastrointestinal tract accumulated into the peritoneal cavity can lead to generalized peritonitis, intraperitoneal abscess formation or it can be drained through the abdominal wound, predisposing to abdominal wall infection and wound dehiscence. Sometimes gravity is not enough for the drainage of the accumulated content and the application of a negative pressure has been proposed as an effective

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tive method for the drainage of anastomotic leaks [2].

Between January 2005 to December 2010, 6 patients who were operated on either electively or as an emergency for gastrointestinal tract malignancies and developed gastrointestinal anastomotic leak, were treated with a simple technique for vacuum drainage of the peritoneal cavity after the diagnosis of gastrointestinal leak. In all cases the edge of either the silicon or the PVC tubes was connected to the vacuum pump system (Basic 30 Aspirator, Medela, Illinois, USA) and -20kPa (-150 mm Hg) negative pressure was applied. The -20kPa vacuum pressure was chosen, since we noticed that greater pressures collapsed the lumen of the silicon tubes. We also noticed that the simple connection of the cannula of an ileostomy

Table 1. Details of the gastrointestinal leaks and the application of the proposed vacuum system

<i>Sex/Age (years)</i>	<i>Brief clinical history</i>	<i>Diagnosis of leak</i>	<i>Period of VS application (days)</i>	<i>Type of fistula finally formed</i>	<i>Healing of the fistula (weeks)</i>
M/86	Disseminated descending colon cancer. Palliative procedure	Enteric content through the intra-operatively placed drain tube and the surgical incision	5	Entero-cutaneous	8
M/54	Splenic flexure colon cancer. Curative operation	Smelly gas but no faecal content through the drain tube	2	Feecal-cutaneous	2
M/51	Disseminated descending colon cancer. Palliative procedure	Enteric content through the lower third of the surgical incision	6	Entero-cutaneous	14
M/62	Cecal adenocarcinoma.	Curative operation Abdominal CT scan revealed a subhepatic abscess infiltrating the right lateral abdominal wall	3	Entero-cutaneous	8
M/75	Pancreatic head adenocarcinoma. Curative operation	Bile through the upper third of the surgical incision	3	Bilio-cutaneous	4
M/77	Rectosigmoid junction cancer. Curative operation	The three lowermost metallic staples for skin approximation were removed leaving a 3cm cutaneous opening	4	Feecal-cutaneous	4

M: males, VS: vacuum system

bag to the vacuum system was similarly insufficient, since the application of even the minimum vacuum pressure (-9kPa) collapsed the bag. Thus, in cases of enteric content drainage through the surgical incision we preferred the air-tightly secure placement of either PVC (N=2) or soft silicon drain tubes (N=1) via the colostomy bag up to the dermal gap and connection of the drain tubes to the vacuum system. With this technique the inflamed, ischemic or necrotic tissues of the abdominal wound were drained into the bag, while the gastrointestinal tract content was driven away of the wound into the vacuum system collector. The vacuum was applied continuously throughout the day and the gastrointestinal content output was recorded every 24 hours. The vacuum was disconnected when less than 30mL of gastrointestinal tract content drainage was recorded within 24 hours and when obvious improvement of the local signs of inflammation was noticed.

Patient characteristics, details of the gastrointestinal leaks and the application of the proposed vacuum system are shown on Table 1. All fistulae were healed spontaneously within 3-14 weeks (median 6). None of the patients developed generalized peritonitis, abscess formation or wound dehiscence. A single fistula was successfully crated in all patients and none of the patients developed adverse events while all abdominal wounds were healed uneventfully.

Vacuum assisted closure (VAC) therapy represents a well-established effective treatment option for the treatment of infected wounds, traumatic open abdominal wounds, wounds with bone exposure, pressure ulcers, diabetic foot ulcers and ulcers arising from venous ectasia in the extremities. The method is contraindicated or should not be applied in unexplored or non-enteric fistulae, in wounds with necrotic tissue, in the treatment of osteomyelitis, over actively bleeding tissues, over wounds

being in contact with exposed blood vessels or organs, in previously irradiated or sutured vessels or organs, or in patients treated with anticoagulants [3]. However, the application of the negative pressure for the management of enterocutaneous fistulae arising within an open abdomen still remains controversial with various reports favoring [4] and other being against its use [5].

In conclusion, the application of low vacuum pressure on the distal end of either the intraoperatively placed silicon drain tubes or the newly placed PVC tubes can desirably lead to an enterocutaneous fistula formation through a single point, disrupting the unfavorable sequences of the leak.

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Frequency of thyroid disease among breast cancer patients: a descriptive study of breast cancer patients

Dear Editor,

Currently, there are numerous studies on the relationship between breast cancer and thyroid disorders. Although the exact mechanism of the relationship remains unclear, many studies have shown that thyroid diseases are common among women with breast cancer [1,2]. Some investigators have suggested increased breast cancer risk in patients treated for thyroid disorders, but others believe that there is no association between thyroid diseases and risk of breast cancer [3]. The purpose of this descriptive study was to examine the frequency of thyroid disease among breast cancer patients and to compare patient and tumor characteristics in patients with and without thyroid disease.

We retrospectively analysed the medical records of breast cancer patients diagnosed between 2004 and 2012 in Hacettepe University, Institute of Oncology.

We examined the frequency of patients with known thyroid diseases including hyperthyroidism, hypothyroidism, Hashimoto's thyroiditis and multinodular goiter and searched patients with suspected thyroid diseases. We evaluated tumor size, tumor grade, estrogen and progesterone receptor status, HER-2 expression and histology of primary tumors. Pearson's chi-square test was used for statistical analysis. A value of $p < 0.05$ was considered statistically significant.

Among 2218 patients with breast cancer, 445 (20.1%) cases with thyroid diseases were found. The majority had multinodular goiter (7.9%, $N=177$) and diffuse goiter (6.9%, $N=153$). The incidence of other thyroid disorders are shown in Table 1. There was no statistically significant difference in patient and tumor characteristics between patients with and without thyroid diseases.

The relationship and coincidence of breast cancer

Table 1. Distribution of thyroid diseases and thyroidectomy in breast cancer patients

<i>Thyroid disease</i>	<i>N</i>	<i>%</i>
Multinodular goiter	177	7.9
Diffuse goiter	153	6.9
Hashimoto's thyroiditis	20	0.9
Thyroid cancer	9	0.4
Toxic goiter	6	0.3
Thyroidectomy	124	5.6

with thyroid disorders is a subject of extensive debate. In a prospective study, prevalence of autoimmune thyroid disorders in Greek breast cancer patients was found to be higher (43.9%) than in patients with benign breast diseases (19%) and healthy controls (18.4%) [4]. Ron et al. reported that there was a significantly elevated risk of thyroid cancer following breast cancer and breast cancer following thyroid cancer [3]. The results of our study also support their conclusion. Our study suggests a fairly frequent occurrence of thyroid disease among breast cancer patients. However, we could not find an association between the presence of a thyroid disorder and any tumor characteristic.

Breast cancer may share some similar etiologic features with thyroid disease. For instance, thyroid and breast, both are under the influence of similar hormones. On the other hand, estrogen may influence the development, physiology and pathology of human thyroid gland

[5]. We did not check all breast cancer patients for thyroid disease, we evaluate only patients with known thyroid disease and patients with suspected thyroid disease so there might be an information bias in our study. Given the inconclusive evidence in the literature we believe that further analytical studies in larger populations are clearly warranted. Meanwhile, we suggest oncologists to check for thyroid disease in women with breast cancer.

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The great surgeon Jean-Louis Faure (1863-1944) and his contribution in the treatment of uterine cancer

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Summary

At the beginning of the 20th century, Professor Jean-Louis Faure, one of the leading surgeons of the innovative Parisian Medical School, published an exhaustive work on uterine cancer. He was the first to perform in France the procedure of total abdominal hysterectomy by median section of the uterus contributing to the evolution of cancer surgery.

Key words: Faure, gynecologic oncology, hysterectomy, surgery



Professeur Jean-Louis Faure

**The eminent cancer surgeon
Jean-Louis Faure.**

Introduction

At the beginning of the 19th century the first hysterectomy attempts ended in bloody failures and the practice was abandoned. In about 1875-1880, the German surgeon Kaltembach reattempts abdominal hysterectomy with operative mortality of around 67%. Therefore, surgeons continued to perform vaginal hysterectomy with low operative mortality (5-10%), but still without chances of recovery.

Thanks to asepsis, we return to abdominal hysterectomy with the Viennese Ernst Wertheim (1864-1920) who proposed in 1900 a radical therapy of uterine cancer. However, between 1896 and 1905, he loses 20 (22%) of his 90 operated patients. Generally, 466 hysterectomies in total were identified in 1907 by Sobre-Casas and performed since 1898 by 27 surgeons, the operative mortality rate being 25.1% (117 deaths) [1].

The surgical technique of hysterectomy was perfected in 1896, thanks to the French surgeons Faure, Terrier, Poirier and Quénu. Actually, the removal of the uterus, called "radical" or "total", with extirpation of neoplastic lesions in the pelvic tissues and partial resection of the vagina increased the survival rates. In 1899, during the 2nd Congress of the International Society of Surgery, some surgeons claimed cure rates about 20 to 40% and absence of recurrence beyond 5 years [2].

Faure's Life - Studies - Career

Jean-Louis Faure was born on October 27, 1863 in Sainte-Foy-la Grande, a small town near Bergerac in Gironde, France, which was the little homeland of the eminent physicians Jean-Martin Charcot (1825-1893), Paul Reclus (1847-1914) and Samuel Pozzi (1848-1918). He died on October 27, 1944 in Saint Émilion. His maternal uncle Paul Reclus was professor of surgery in Paris. Faure began his studies in the Protestant College of Sainte-Foy and graduated in the Lyceum Louis-le-Grand in Paris. In 1884, he enrolled in the Faculty of Medicine (Photo). In 1886, he becomes extern in the department of Alexis Legroux (1839-1894), professor of pathology in the Laennec Hospital and in 1887-1890 he becomes an intern. On July 5, 1888, Faure married Madeleine Bourgeois. From this marriage four children were born.

In 1889-91, he was appointed assistant in anatomy and later on lecturer. In 1895, he became hospital physician. In 1898, he obtained the title of associate professor of surgery. In 1899-1904, he was appointed lecturer of the surgical clinic at the Hôtel-Dieu hospital. In 1918, he was designated lecturer of clinical gynecology and in 1919 he replaced the late professor Samuel Pozzi in the chair of clinical gynecology at Broca Hospital. In 1934, he became emeritus professor of surgery.

In 1924, Faure received the insignia of Commander of the Legion of Honor and became member of the French Academy of Medicine. One year later he was elected president of the Surgical Society [3].

Faure's scientific work

Jean-Louis Faure was one of the greatest surgeons of his time. Although he was specialized in gynecology, he presented an interesting work on cancer and left his mark in many surgical techniques such as the extirpation of the parotid glands and the drainage of peritonitis [3].

His name is related to: 1) Faure's needle: needle of round point handle, for ligation of the hypogastric artery. 2) Faure's needle with a lever: variety of lateral Reverdin needle with lever. 3) Faure's operation: surgical technique of subtotal abdominal hysterectomy by uterine hemisection. 4) Faure's clamp: long curved hemostatic forceps. 5) Faure's extra-condylar veins [4]. 6)

Faure's and Ionesco vestibule (pre-vestibular funnel): inconstant prolongation of the vestibule of the back cavity of the omentum, to the right of the opening into lesser sac of peritoneum [5].

His publications include: his doctoral thesis entitled 'The suspensory apparatus of the liver (L'appareil suspenseur du foie), Hepatoptosis and hepatopepy (L'hépatoptose et l'hépatopéxie) published in 1892, Surgery of the Uterine adnexa (Chirurgie des annexes de l'utérus) printed in 1902, Clinical lessons and operative techniques (Leçons de clinique et de techniques opératoires), his famous book Hysterectomy (L'hystérectomie) published in 1906 and Treatise on medical and surgical gynecology (Traité de gynécologie médico-chirurgicale) appeared in 1911 [6].

His work on oncology

In 1896, Faure practiced the first successful hysterectomy in a cancer patient. It was the first operation of its kind performed in France.

In his book entitled *Chirurgie des annexes de l'utérus*, Faure describes the procedure of total abdominal hysterectomy by median section of the uterus. He points that it is an extremely simple procedure without any risk of damaging the ureter. The surgeon can reverse the uterus giving in the bottom of the pelvis space to maneuver and to reach the adnexa below by taking them off upwards, both right and left. According to Faure, this method collects all the facilities and is the ideal of operative simplicity.

Faure actually improved this surgical technique so that in 1920 he achieved long-term survival in 60% of the cases. He also claimed that hysterectomy was the treatment of choice in cancer of the cervix when the uterus was still mobile [7].

In 1932, he stated that he had successfully removed uterine cancer in 86% of the cases. However, these results involved mainly early-stage uterine cancers.

Moreover, to relieve the suffering of his patients professor Faure in 1891 and professor Mathieu Jaboulay in 1901 used to practice section of the spinal nerves, in order to cut off the sources of pain. These caused such physiological alterations though, that they had to abandon this method [8].

Faure was also involved in the 19th century's debat-

ing issue of cancer contagiousness. He and other great oncologists of his time like Roussy and Delbet did not attach the least importance on this belief, rejecting that theory [8].

Conclusion

Professor Faure revolutionized the practice of hysterectomy opening new horizons of research in cancer surgery. In fact, it was not until the aftermath of the Second World War when the modern fight against cancer begins with the discovery of new chemotherapeutic agents.

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Acknowledgements

All other persons who contributed to the work should be listed in “Acknowledgements”. These include people who provided purely technical help, writing assistance, or general support. All financial support should be clearly acknowledged as well as any detail regarding the role of the funding organization in the creation of the manuscript.

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