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ORIGINAL ARTICLE

MDM2 344T>A polymorphism; could it be a predictive marker of anthracycline resistance?

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Summary

Purpose: *To find a possible association between the Mouse Double Minute 2(MDM2) 344T>A, alone and in combination with p53 72 Arg/Pro polymorphism, and resistance to anthracycline-based chemotherapy of breast cancer in Tunisia.*

Methods: *This study enrolled 542 patients with invasive ductal carcinoma (IDC) treated with anthracycline-based chemotherapy. Genomic DNA was isolated from whole blood, using the phenol chloroform method. Anthracycline response was scored according to the World Health Organization (WHO). MDM2 344T>A polymorphism was genotyped using real time polymerase chain reaction (RT-PCR) with the TaqMan method. Data was statistically analyzed using the x^2 test.*

Results: *Response was evaluated in 400 patients, of whom a quarter was found to be resistant to chemotherapy. Genetic data revealed that resistance to anthracycline-based chemotherapy did not seem to be correlated with 344T>A polymorphism in the studied population. Also, no significant association was found between the single nucleotide polymorphism (SNP) 344T>A status and clinicopathologic parameters (p>0.05 for all comparisons). Moreover, analysis of p53 rs1042522 and MDM2 rs1196333 combination showed no significant association between these two genetic variants and anthracycline resistance (p=0.2).*

Conclusions: *Our findings provide no evidence indicating that SNP 344 T>A may affect response to anthracycline-based chemotherapy. However, the results obtained from the combination of SNPs 344T>A of MDM2 and 72 Arg/Pro of p53, do not support the hypothesis of the prominent role of common p53 and MDM2 variations in the genetic mechanisms of chemotherapy resistance in breast cancer.*

Key words: *anthracycline, breast cancer, MDM2 344T>A, P53 72Arg/Pro, resistance*

Introduction

Anthracyclines are among the most commonly applied antitumor drugs ever developed [1] for the treatment of a wide range of cancer types, including non-Hodgkin and Hodgkin's lymphoma, multiple myeloma, lung, ovarian, gastric, thyroid, breast, sarcoma and pediatric cancers [2,3]. Several mechanisms have been proposed to explain the anthracyclines' antitumor activity [4], but mainly their mechanisms of action are ascribed to their intercalative interaction with DNA, leading to inhibition of topoisomerase II activity and apoptosis [5]. The major limitation of the usefulness of anthracyclines is the development of resistance [6], which can occur prior to drug treatment (primary or innate resistance), or may develop over time following exposure (acquired resistance) [7]. Key questions for molecular oncologists, clinicians and patients include, "what are the mechanisms of intrinsic or acquired drug resistance in solid tumors, and how might they be circumvent-

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ed? ". Numerous factors influence the ability of a drug to kill cancer cells [8]. To date the most widely studied cellular mechanisms of tumor resistance are those associated with drug efflux mechanisms, involving members of the Adenosine Triphosphate (ATP)-Binding cassette, ABC membrane transporter family notably p-glycoprotein (P-gp), multidrug-resistant protein1 (MRP 1) and breast cancer resistance protein (BCRP) [5]. However, there are other mechanisms that are also deemed to be important, such as disruptions in apoptotic signaling pathways. Many studies focused on the *p53* pathway, which is considered as the major target of anthracyclines. Our group has previously evaluated the relationship between resistance to anthracycline and SNP72 Arg/Pro of *p53*. Neither of the alleles of *p53* polymorphism were found to be associated with this resistance [9]. This observation made us hypothesize that anthracyclines' resistance could be due to inactivation of other genes acting up or a downstream in the p53 functional pathway [10,11].

The search for genetic variations in the *p53* pathway begun by looking in the *MDM2* (Mouse Double Minute 2 homolog) gene, which encodes an important negative regulator for p53 [12], located on chromosome 12q13-14 [13]. In many cellular processes, *MDM2* acts as a key negative regulator of p53 through directing binding, ubiquitination, and degradation of p53 [14]. Many cancer cells display high levels of *MDM2* expression, resulting in rapid cancer progression and lack of response to therapy in a subset of human cancer types [15]. SNPs can generate biological variations between people by causing differences in the recipes for proteins that are written in genes. These differences can in turn influence gene functions and clinical phenotypes [16]. Currently, 2 functional SNPs, SNP 309 (rs2279744) [12] and its antagonist SNP 285 (rs117039649) [17], have been reported to enhance and decrease *MDM2* gene expression, respectively. SNP 344T>A (rs1196333) is the third *MDM2* promoter polymorphism, located 35 base pairs downstream of SNP 309 [18] , and recently identified as an important variant that could influence the expression of *MDM2* gene through the modulation of transcription factor binding [19]. However, this SNP did not have a clear function. The associations between this SNP and cancer risk have been evaluated in different types of cancers (ovarian, breast, endometrial, prostatic and hepatocellular carcinoma) [18,19]. However, until now, only one study has investigated the effect of this SNP and chemotherapy resistance [20]. Because of the aforementioned fact, the objective of our study was to find a possible association between *MDM2* 344T>A polymorphism and anthracycline-based chemotherapy resistance and to assess the potential contribution of the common functional gene variants p53 72Arg/Pro and *MDM2* SNP344 T/A, to the genetic susceptibility to anthracycline resistance.

Methods

Patients

Between January and June 2013, we enrolled 542 histologically confirmed breast cancer patients, treated at our institute (Salah Azaiz Institute). Consent for participating in the study and for personal data management was obtained from all patients. Age at diagnosis, family and personal history of breast cancer, age at menarche, marital status, age at first live birth, menopausal status, tumor characteristics, chemotherapeutic agents used, number of cycles given and response to chemotherapy were evaluated by reviewing medical files. Patients were considered appropriate if they had completed all the anthracycline-based chemotherapy regimens, while those who had not been treated yet, received any form of chemotherapy without anthracyclines or not completed all regimens were excluded. Usually anthracycline-based chemotherapy consists of 5-Fluorouracil (5-FU), the anthracycline compound and cyclophosphamide (FAC- FEC- EC or AC regimens).

A response evaluation was performed after the first two courses of chemotherapy and every two courses thereafter, according to established WHO criteria [21].

Evaluation of chemotherapy response

Therapeutic response after anthracycline-based chemotherapy was evaluated in 400 patients and was scored according to the World Health Organization (WHO). In the absence of clinical evidence of tumor in the breast, response to therapy was categorized as complete clinical response (CR), as partial remission (PR), if the reduction of tumor volume exceeded 50%. Tumor remission less than 50%, or an increase of tumor volume up to 25%, was scored as stable disease (SD). An increase of more than 25% or appearance of new lesion(s) was recorded as progressive disease (PD) [22]. In this study, responsive patients were considered those who showed CR or PR, whereas those with SD or PD were classified as non-responders or resistant. We defined 2 subgroups of patients with anthracycline-resistant disease. Primary anthracycline resistance was

defined as PD while receiving neoadjuvant, first or second line anthracycline-containing chemotherapy. Secondary resistance was defined as initial response followed by PD (recurrence or metastases), within 9 or even 12 months, after completion of neoadjuvant or adjuvant therapy or first-line, containing chemotherapy for metastatic disease.

Genotyping of MDM2 polymorphism

Five milliliters of venous blood were collected in a sterile tube containing EDTA and stored at -80°C. Genomic DNA was isolated from leucocytes, using the phenol-chloroform method [23] and stored at 4°C until use. Concentration and purity of the DNA were verified by a spectrophotometer (SINNOWA ER500, USA). The absorbance ratio at 260/280 nm of all the samples ranged from 1.8 to 2, indicating they were all free from contaminants. This control enabled us to consider all the DNA samples suitable for RT-PCR assay. RT-PCR analysis was performed with Step One (Applied Biosystems, HTDS, Tunis, Tunisia). Predesigned and validated gene specific probe-based TaqMan genotyping assays from Applied Biosystems were used for the target study gene (rs1196333). Every set contained gene-specific forward 5'-CCCGGACGATATTGAACA-3' and reverse primer 5'-AGAAGCCCAGACGGAAAC-3' as well as fluorescence labeled probes. Reactions were performed using the TaqMan Universal PCR Master Mix (HTDS, Tunis, Tunisia) and each reaction was plated into 48-well plates. The amplification profile was one cycle of denaturation for 30 s at 60°C, followed by 40 cycles of 15 s at 95°C and annealing extension for 1 min at 60°C.

Statistics

Statistical Package for Social Science (SPSS Inc, Chicago, Ill, USA) advanced models 20.0 software was used for the statistical analyses. Data is presented as N (%). Genotype frequencies and association with other clinical parameters were calculated using the x^2 test with Yates' corrections. Statistical significance was set at $p<0.05$.

Results

Patient characteristics

A total of 542 patients with breast cancer were enlrolled in the study. For 74% of the cases (N=400), the response of the anthracycline-based chemotherapy data was available, which was a prerequisite for inclusion in the present study. Patient and tumor characteristics of 400 cases are summarized in Table 1. All the participants were female. The median age was 48 years (range 20-80). Fifty-six of the cases (14%) had a family history of breast cancer. Thirty-four of the 56 had first degree relatives and 22 had only second degree relatives with breast cancer. According to TNM

Table 1. Patient and tumor characteristics

Characteristics	
Number of patients, N (%)	400 (100)
Median age, years (range)	48 (20-80)
Family history of breast cancer (%)	
Yes	14
No	86
Personal history of breast cancer (%)	8.5
Yes	8.5
No	91.5
Median age at menarche (years)	13
Marital status (%)	
Married	89
Unmarried	11
Median age at first live birth (years)	24
Menopausal status (%)	
Premenopausal	52
Postmenopausal	48
Breast (%)	
Right	44
Left	56
Histopathological type (%)	
Invasive ductal	100
Other	0
T stage (%)	
T1- T2	54
T3- T4	46
Clinical node status (%)	
N+	76
N-	24
Distant metastasis (%)	
M0	93
M1	7
Grade (%)	
I	9
II	65
III	26
Hormone receptor status (%)	
Negative	29
Positive	71

Table 2. Different therapeutic settings

Settings	%
Chemotherapeutic regimen	
Adriamycin	23
Epirubicin	77
Therapeutic approach	
Neoadjuvant	29
Adjuvant	66
Palliative	5
Median number of chemotherapeutic cycles, N (range)	5 (1-8)
Clinical response (%)	
CR+PR	76
SD+PD	24
Anthracycline resistance	
Primary	63
Secondary	37

For abbreviations see text

classification system (UICC), most patients were diagnosed with stage II or stage III breast cancer. Pathological examination showed invasive ductal carcinoma in 400 patients (100%). Immunohistochemistry revealed that 284 (71%) patients were positive for hormone receptors.

Chemotherapy regimens

All patients received anthracycline-based chemotherapy, which included FEC (5-FU 500 mg/m^2, epiribucin 100 mg/m^2 and cyclophosphamide 500 mg/m^2), FAC (5-FU 500 mg/m^2, doxorubicin (adriamycin) 50 mg/m^2 and cyclophosphamide 500 mg/m^2), EC (epirubicin 100 mg/m^2 and cyclophosphamide 500 mg/m^2) or AC (doxorubicin 50 mg/m^2 and cyclophosphamide 500 mg/m^2) every 3 weeks. Twenty-three percent of the patients received adriamycin and 77% received epirubicin. The choice of chemotherapy protocols depended on the availability of the agents at the time of indication. Patients received a maximum of 8 cycles of chemotherapy. The median number of treatment cycles received was 5 (range 1-8). Variations in the number of treatment cycles were caused by different reasons, including prohibitive toxicity, disease progression while on therapy and disease stabilization after 4 cycles. Patients were divided according to the type of therapeutic approach into the neoadjuvant (29%), adjuvant (66%) and palliative (5%) group (Table 2).

Response to anthracyclines

Clinical data revealed that among 400 patients, a quarter (24%) was resistant to anthracycline-based chemotherapy. Within the study population, we defined 2 subgroups of patients with anthracycline-resistant disease. Sixty-three percent of patients had primary anthracycline resistance and 37% had secondary anthracycline resistance as illustrated in Table 2.

Genotyping

SNP344 status and response to anthracycline-based chemotherapy

The association between the polymorphic variants and response to anthracycline-based chemotherapy was carefully analyzed (Table 3). For the chemoresistance and chemosensitive cohorts SNP 344 was observed at frequencies of TT= 88% and 92%; TA= 7% and 5%; AA= 5% and 3%, respectively. Minor differences observed between the 2 groups of patients were not statistically significant, indicating that SNP344 is not a predicative factor for anthracycline resistance in breast cancer.

SNP344 T>A status and clinicopathologic parameters

MDM2 rs1196333 was successfully gen-

Table 3. Allele frequencies and genotype distribution of MDM2 344T>A polymorphism in breast cancer patients

	Chemoresistant N=95	*Chemosensitive N=305*	*p value*
T allele	174	578	>0.1
A allele	16	32	
Genotype			
TT	84	281	
TA	6	16	>0.3
AA	5	8	

Table 4. The relationship between SNP344 T>A genotypes and different clinical parameters in the study population

Variables	*Genotypes*	*N (%)*		*p value*
Total N=400	TT	365 (91)		
	TA	22 (5.5)		
	AA	13 (3.5)		
Tumor localization		Right breast (N=184)	Left breast (N=216)	
	TT	166 (90)	199 (92)	0.704
	TA	12 (6.5)	10 (5)	
	AA	6 (3.5)	7 (3)	
		T1-T2 (N=216)	T3-T4 (N=184)	
Depth of invasion	TT	198 (92)	167 (91)	0.858
	TA	12 (5)	10 (5)	
	AA	6 (3)	7 (4)	
		N+ (N=304)	N- (N=96)	
Lymph node metastasis	TT	278 (91)	87 (91)	0.584
	TA	15 (5)	7 (7)	
	AA	11 (4)	2 (3)	
		GI (N=36)	GII-GIII (N=364)	
SBR grade	TT	33 (92)	332 (91)	0.06
	TA	0	22 (6)	
	AA	3 (8)	10 (3)	

SBR: Scarff Bloom Richardson grade

otyped in all study subjects. The frequency of MDM2 rs1196333 genotypes was 91% (N= 365) for the TT variant, 5.5% (N=22) for the TA variant and 3.5% (N=13) for the AA variant (Table 4). We examined SNP 344 genotype with respect to a number of clinicopathologic features. From Table 4, it is clear that the distribution of tumor localization, depth of invasion, lymph node metastasis and tumor grade were not significantly different among the polymorphic variants. These results suggested that SNP344 T>A didn't have a prognostic value in breast cancer.

Combination of MDM2 rs1196333 T>A and p53 rs1042522 G>C

The breast cancer patients analyzed, were enrolled in the prospective study aiming at identifying genetic mechanisms of resistance to anthracycline-based chemotherapy; this study evaluated the relationship between resistance to anthracycline and SNP 72Arg/Pro of p53 [9]. We assessed the impact of SNP344 T>A in response to anthracycline-based chemotherapy among individuals harboring p53 SNP72 Arg/Arg, Arg/Pro and Pro/Pro. We did not find any association between the SNP344 status and resistance to anthracycline. Moreover, the united analysis of these two polymorphisms showed that, when compared to individuals carrying rs1196333 TT and rs1042522 GG (the frequency was 93% [14/15] in the chemoresistants' group and 73% (30/41) in the chemosensitives' group) and carriers with rs1196333AA and rs1042522 CC genotypes (the frequency was 7% [1/15] in the chemoresistants' group and 27% (11/41) in the chemosensitives' group), no significant association with resistance to anthracycline was found (p= 0.1) (Table 5).

Discussion

Anthracyclines are the most active and widely used chemotherapeutic agents for breast cancer [24]. Their general effects are believed to require a functioning apoptotic pathway to induce cell death [25]. The major limitation of the usefulness of anthracyclines is the development of resistance [6]. We have learned in the treatment of cancer, that "One size doesn't fit all"; thus, each individual solid tumor, in each person, is unique, in cause, rate of progression and responsiveness

Table 5. Distribution of genotype combinations of the SNPs 72 G/C of p53 and 344 T>A of MDM2 among breast cancer patients

	Chemoresistant (N=95) N (%)	*Chemosensitive (N=305)* N (%)	*p value*	*p value (TT/GG) vs (AA/CC)*
TT/GG	14 (14)	30 (10)		
TT/GC	34 (36)	130 (43)		
TT/CC	40 (42)	117(38)		
TA/GG	1 (1)	4 (1)		
TA/GC	2 (2)	12 (4)	0.2	0.1
TA/CC	2 (2)	1 (0.3)		
AA/GG	0	0		
AA/GC	1 (1)	0		
AA/CC	1 (1)	11 (3.7)		

to surgery, chemotherapy and radiation therapy [26,27]. Identification of patients with non-responsive breast cancer, so their therapy could be individualized, is a hot topic in breast cancer research [28]. Personalization of treatment should take into account the individual genetic characteristics, borne by gene polymorphisms [29]. Given its involvement in apoptosis pathway, *MDM2* was a subsequent candidate for evaluation, as a predictive biomarker of anthracycline resistance. *MDM2* controls processes like growth, arrest, senescence and apoptosis [30-34]. Naturally occurring sequence variations in the *MDM2* promoter region change expression of MDM2 protein and affect p53 tumor suppression [12]. SNP344T>A (rs1196333) is an *MDM2* promoter p2 polymorphism [19], located 35 bp downstream of SNP309 [18] and it was recently identified as an important variant that could influence the expression of the *MDM2* gene, through the modulation of transcription factors binding [19]. However, this SNP did not have a clear function. A large number of studies have investigated the role of the functional 344T>A polymorphism in the modulation of cancer risk in different types of cancers (ovarian, breast, endometrial, prostatic and hepatocellular carcinoma) [18,19]. However, up until now only one study has investigated the effect of this SNP in response to chemotherapy [18]. It is therefore of interest to investigate the association between SNP344T>A of *MDM2* and resistance to anthracycline-based chemotherapy in different therapeutic approaches (neoadjuvant, adjuvant and palliative) and in a large series of breast cancer, using the TaqMan SNP genotyping assay. We didn't find any statistically significant association between *MDM2* 344T>A polymorphism and resistance to anthracycline-based chemotherapy. This finding is in keeping with the previous observation of Knappskog et al. [18], assessing the effects of the rs1196333 status on the response of the neoadjuvant chemotherapy in 307 breast cancer patients; 106 patients were evaluated for the impact of this SNP on response to doxorubicin monotherapy or to combined cyclophosphamide, methotrexate and 5-FU (CMF), while 201 patients were evaluated for epirubicin and paclitaxel monotherapy. The results showed that SNP344T>A status does not affect the response to either DNA damaging drugs (doxorubicin, mitomycin) or spindle poison (paclitaxel) (p>0.1 for all comparison) [18]. We therefore assessed the potential impact of SNP344 status on several clinical parameters. The distribution of tumor localization, tumor size, lymph node status and Scarff Bloom Richardson (SBR) grade was not significantly different among the polymorphic variant. These results suggested that SNP344T>A didn't have either a predictive or a prognostic value in breast cancer in our population. Knappskog et al. [18] assessed the potential impact of SNP344T>A status on the age of onset in endometrial, prostate, breast and ovarian cancer. No effect was found on these four cancer types. Anthracyclines kill tumor cells by activating the common apoptotic pathway, thus inactivation of genes in the same pathway may be a mechanism causing resistance. We therefore assessed, for the first time, the impact of SNP344T>A on the response to anthracycline-based chemotherapy among individuals harboring SNP72 Arg/Arg, Arg/Pro and Pro/Pro of p53, as the two variants under investigation are known to be functionally coupled. Collectively, the results we obtained from our large cohort,

do not support the hypothesis of the prominent role of common p53 and MDM2 variation in the genetic mechanisms of chemotherapy resistance in breast cancer.

In conclusion, our findings provide no evidence indicating that MDM2 344T>A polymorphism status, alone or in combination with *p53* SNP72Arg/Pro, may affect response to anthracycline-based chemotherapy. A SNP309 T>G (rs227944) and its antagonist 285G>C (rs117039649) are found in the MDM2 promoter and have been reported to enhance and decrease MDM2 gene expression respectively and affect the risk of multiple cancer types [12,17,20,35]. However, SNP344 A resides on the SNP 309 T allele [18]; it is therefore of interest to assess the impact of the three MDM2 SNPs together on the response of anthracycline-based chemotherapy. There is some hope that understanding a collection of SNPs in patients may someday help in the prevention and the treatment of this disease. It may permit a better selection of agents for chemotherapy and predictions for the therapeutic outcome.

Conflict of interests

The authors declare no confict of interests.

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