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ORIGINAL ARTICLE

Research on and clinical importance of duplications in various chromosomal regions in addition to Philadelphia chromosome in chronic myeloid leukemia

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Summary

Purpose: *To investigate the chromosomal aberrations in chronic myelogenous leukemia (CML), particularly in chromosomal regions which carried 67 genes pertaining to oncogenes, transcription factors, signal transduction, tumor suppressors, apoptosis etc, in addition to Philadelphia (Ph+) chromosome by multiplex ligation-dependent probe amplification (MLPA) method and to compare them with clinical parameters.*

Methods: *The aberrations were investigated in 48 CML patients receiving imatinib therapy and a group of 15 healthy controls, by using the MLPA method between 2000 and 2009. The obtained results were compared both between patient and control groups and with clinical parameters.*

Results: *Duplication was detected in the fibroblast growth factor receptor 1 (FGFR1) gene of 2 patients, inosine 5'monophosphate dehydrogenase 1 (IMPDH1) gene of 4, postmeiotic segregation increased S. Cerevisiae 2 (PMS2) gene of 1, nuclear factor kappa beta (NFKB) of 5 and T-cell translocation 2 (LMO2) gene of 1 patient. Univariate analysis showed that splenomegaly, advanced age, Sokal risk score (SRS) and the duplications in IMPDH1 and FGFR1 genes significantly shortened 7-year event-free survival (EFS); multivariate analysis showed that only the duplications in IMPDH1 and FGFR1 genes were the factors that significantly affected EFS. No statistically significant correlations were detected between duplications and other clinical parameters.*

Conclusion: *Duplications in 4 genes (FGFR1, IMPDH1, PMS2, LMO2) in addition to Ph+ chromosome in CML patients were detected for the first time. This study indicates that chromosomes 7 and 8 should be particularly investigated in more detail in addition to the Ph+ chromosome for better determination of disease prognosis and selection of alternative treatments.*

Key words: chronic myelogenous leukemia, DNA, MLPA, PCR, prognosis

Introduction

CML, also known as chronic granulocytic leukemia, originates from abnormal hematopoietic stem cells and is a clonal myeloproliferative disease that affects myeloid, erythroid, monocytic and megakaryocytic series [1]. Overproliferation of the myeloid series in the bone marrow is characterized by leukocytosis and splenomegaly based due to increase in mature myeloid series in the peripheral blood [1]. CML has a privileged feature in that the Ph+ chromosome plays a role in its pathogenesis and can be treated with interferon (IFN) [2]. The Ph+ chromosome is formed via a balanced reciprocal translocation t(9;22)(q34;q11) between the long arms of chromosomes 9 and 22 [3,4]. This reciprocal translocation takes place when the Abelson (ABL) oncogene, located on chromosome 9 and having a tyrosine kinase activity, and the BCR (breakpoint cluster region) gene, located on chromosome 22, come side by side as of the breakpoint. The breakpoint in the ABL gene generally involves the 5' (towards the centromere) edge of exon 2. ABL exons 2 and 11 (a2-a11)

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settle in the breakpoint (major breakpoint cluster region; M-BCR) between exons 2 and 16 (b1-b5) of the BCR gene. As a result, the gene fusion that takes place results in the formation of BCR-ABL chimeric gene and the chimeric proteins encoded by this gene, i.e. $p190^{BCR-ABL}$, $p210^{BCR-ABL}$ and $p230^{BCR-ABL}$ (190, 210 and 230 kd, respectively). Through autophosphorylation, these proteins activate cell proliferation, maturation, apoptosis and adhesion signal pathways, thereby leading to malignant cell transformation [1,3].

Therapies such as INF, imatinib or stem cell transplantation are used in the treatment of CML. The positive therapeutic effects of INF in cases in accelerated or blastic phase, besides the resistant chronic-phase CML cases, made this cytokine the first therapeutic approach. It stimulates and reinforces the immune system against the malignant cells, and slows down their proliferation [5]. Imatinib removes the enzyme activity of the BCR-ABL fusion protein that occurs when the Ph+ chromosome leads to an increase in leukocytes and inhibits the proliferation of leukocyte cells in CML. In addition, it inhibits the thrombocyte-derived growth factor receptor and stem cell factor [6]. In CML, stem cell transplantation is performed in patients who do not respond to INF or imatinib therapy and is carried out by obtaining stem cells from bone marrow or peripheral blood either from a sibling with a matching HLA tissue group or, if no sibling is available, from a non-relative donor with a matching tissue group [7].

MLPA is a method that was first developed by Schouten et al. in 2002. With this method some 40 different DNA sequences can be studied using only 20 ng of human DNA, and it is possible to characterize exon deletions and duplications, trisomies and chromosomal aberrations in cell lines and tumor specimens and to detect single nucleotide polymorphisms (SNPs) mutations [8]. There are some 977 studies that used this method, the results of which have been published and registered in PUBMED. Of these manuscripts, 322 are studies that have been performed in association with various malignancies (e.g. breast, ovarian, colorectal, duodenal cancers and lymphatic leukemia). No studies performed with the MLPA method in association with CML have been available in the literature yet [9].

In this study we aimed to investigate the chromosomal aberrations in CML, and particularly the aberrations in chromosomal regions which carried 67 genes pertaining to oncogenes (such as EMS1, HRAS, MYC and NRAS), transcription factors (such as RENT2 and NFKBIA), signal transduction (such as CDKN1B, SPG3A, IMPDH1, FGFR1 and PTPRD), cytokines (such as IL1, TANK and IL13), immune system (TGFBR1 and PTP4A3), tumor suppressors (BRCA1, MDM2, BRCA2 and RB1) and apoptosis (such as MOAP1, BAX and PDCD8), in addition to the Ph+ by the MLPA method and to compare them with clinical parameters.

Methods

Forty-eight Ph+ (p210 chimeric protein) CML patients (17 men and 31 women) receiving imatinib therapy were studied at the Department of Hematology, in the Faculty of Medicine at Gaziantep and Erciyes Universities. The study was approved by the local institutional ethics committee and performed according to the Declaration of Helsinki of 1975.

DNA samples were isolated from blood samples obtained from the CML patients and 15 healthy controls using the DNA isolation kit (Invitek Cat No: CA070023, USA) according to the manufacturer's protocol. MLPA was performed using two specifically designed sets of probes for testing genomic aberrations in CML (P005 and P253, MRC-Holland, Amsterdam - The Netherlands). The complete MLPA protocol, including hybridization, ligation, and PCR, was performed according to the manufacturer's instructions. Amplification products were identified and quantified by capillary electrophoresis on an ABI 3100 genetic analyzer and analyzed using an ABI GeneMapper software package, version 2 (Applied Biosystems, Foster City, CA) [8]. The obtained results were compared both between patient and control groups and with clinical parameters such as SRS [10] (patient's age, spleen size, percent of blasts and platelets), hemoglobin, leukocyte and thrombocyte counts, advanced age, sex and splenomegaly, and their effects on overall survival were evaluated. The relative risk for disease progression was calculated as low, intermediate or high according to the SRS and assessed by a web-based calculator (http://www.roc.se/hematologi/KLM/Sokal.asp).

Statistical analysis

Data analysis was made using the SPSS for Windows software (version 13.0; SPSS, Chicago, IL). The statistical significance of the differences between the patient and the control groups was estimated by logistic regression analysis. Adjusted odds ratios (ORs) were calculated with a logistic regression model that controlled for gender and age and reported with 95% confidence intervals. Survival probabilities were estimated by the Kaplan-Meier method and differences were compared using the log-rank test [11]. The Cox stepwise regression analysis was employed to confirm the significance of risk factors [12]. In multivariate analysis, we used eliminated stepwise variables with < 10% significance. p values <0.05 were considered to indicate statistical significance.

Results

The clinical characteristics of 48 CML patients included in the study are shown in Table 1. The median age was 43 years (range 20-74). Median overall and EFS durations have not been reached yet (7-year overall survival probability 98%; 7-year EFS probability 64%). Gender, age, SRS, splenomegaly, hemoglobin, thrombocyte count, initial treatment and du-

Table 1. Clinical features of CML patients in chronic phase

	N (%)
Number of patients	48
Age at diagnosis, years, median (range)	43 (20-74)
Age ≥ 60 yrs	6 (12.5)
Male/female	17 /31 (35.4/64.6)
Splenomegaly	32 (66.7)
Hemoglobin 12<g/dL	35 (72.9)
Leukocytes > 50 × 10^9/L	32 (66.7)
Platelets > 450 × 10^9/L	25 (52.1)
Sokal risk score at diagnosis	
Low	8 (16.7)
Intermediate	22 (45.8)
High	18 (37.5)
Initial treatment	
Imatinib 400 mg/d	42 (87.5)
Interferon-α→imatinib 400 mg/d	6 (12.5)
Response ELN criteria (18 mo)	
Optimal	33 (68.7)
Suboptimal	9 (18.8)
Failure	6 (12.5)
Mortality	1 (2.1)
Event*	12 (25)
Chromosomal abnormalities in addition to Ph+	3 (6.3). Trisomy 8, Monosomy 7, Trisomy 21
Time after diagnosis, mo (range)	48.2 (14.9-168.4)
Duration of imatinib, mo (range)	37.5 (14.9-103.4)

ELN: European Leukemia Net, mo: months, *death (n=2) due to progression to accelerated phase or blastic phase (n=2), loss of major cytogenetic response (n=8), CML: chronic myeloid leukemia

plications, which were all among the factors affecting the 7-year overall survival, showed no statistical significance. The 7-year EFS was 40% in 6 patients aged over 60 years and 68% in 42 patients aged below 60 years (p=0.039). According to SRS, the 7-year EFS was 100% in 8 low-risk patients, 74% in 22 intermediate-risk patients and 25% in 18 high-risk patients (p=0.028). It was 44% in 32 patients with splenomegaly, and 100% in 16 patients without splenomegaly (p=0.005) (Table 2).

Duplication was detected in the FGFR1 gene of 2 patients, IMPDH1 gene of 4 patients, PMS2 gene of 1 patient, NFKBI gene of 5 patients and LMO2 gene of 1 patient (Table 3), whereas no duplication was detected in the healthy control group. Univariate analysis showed that splenomegaly, advanced age, SRS and the duplications in IMPDH1 and FGFR1 genes significantly shortened the 7-year EFS, and when these parameters were evaluated in multivariate analysis, only the duplications in IMPDH1 and FGFR1 genes were the most important factors affecting EFS (Cox regression analysis p=0.028) (Table 4; Figures 1 and 2). No statistically significant correlations were detected between the duplications and other clinical parameters.

Table 2. Univariate analysis (log-rank test) of duplication in patients with chronic-phase chronic myeloid leukemia

	N	*7-yr OS %*	*log-rank p-value*	*7-yr EFS %*	*log-rank p-value*
All patients	48	98		64	
Gender					
Female	31	96		59	
Male	17	100	0.464	72	0.981
Age (years)					
<60	42	97		68	
≥60	6	100	0.717	40	0.039
Sokal risk score at diagnosis					
Low	8	100		100	
Intermediate	22	100	0.355	74	0.058
High	18	93		25	
Low-intermediate/High	30/18	100/93	0.150	79/25	0.028
Splenomegaly					
Yes	32	96		44	
No	16	100	0.464	100	0.005
Hemoglobin (g/dL)					
<12	35	97		53	
≥12	13	100	0.534	73	0.366
Leukocytes (×10^9/L)					
<50	16	100		74	
≥50	32	97	0.487	55	0.348
Platelets (×10^9/L)					
<450	23	100		68	
≥450	25	96	0.351	61	0.685
Duplication					
Group 1 (NFKB1, LMO2, PMS2)	6	100		100	
Group 2 (IMPDH1, FGFR1)	6	100		0	
No duplication	36	97	0.842	68	0.024
Group 2 (IMPDH1, FGFR1) /other	42/6	97	0.842	73/0	0.012

OS: overall survival, EFS: event free survival, yr: years

Table 3. Characteristics of patients with duplication

Patient no.	*Age (years)*	*Sex*	*Sokal risk score*	*Hemoglobin at diagnosis ($\times 10^9$/L)*	*Leukocytes at diagnosis ($\times 10^9$/L)*	*Duplications*	*Response ELN criteria*	*EFS (mo)*	*OS (mo)*
1	54	F	1.09 (Intermediate)	10.3	178	FGFR1	Failure	34.6 (accelerated phase)	51.5 (alive)
2	51	F	0.82 (Intermediate)	12	81	NFKBI	Optimal	82.1	82.1 (alive)
3	67	F	1.13 (Intermediate)	8.5	91	IMPDH1	Optimal	34.0	34.0 (alive)
4	62	F	1.03 (Intermediate)	9.5	88	NFKBI	Optimal	38.0	38.0 (alive)
5	58	F	0.79 (Low)	8.5	76	IMPDH1	Optimal	36.5	36.5 (alive)
6	46	F	0.98 (Intermediate)	10	44	NFKBI	Optimal	40.7	40.7 (alive)
7	49	F	1.12 (Intermediate)	13.2	28	IMPDH1	Optimal	0.5 (loss of MCyR)	40.5 (alive)
8	34	F	1.11 (Intermediate)	8.6	88	IMPDH1	Failure	12.9 (blastic phase)	26.8 (alive)
9	74	M	1.46 (High)	10	43	NFKBI	Optimal	20/01/12	20.1 (alive)
10	53	F	1.02 (Intermediate)	9.5	86	LMO2	Optimal	59.7	59.7 (alive)
11	54	M	1.94 (High)	12.8	243	PMS2	Optimal	26/07/12	26.7 (alive)
12	54	F	1.15 (Intermediate)	9	56	FGFR1	Optimal	22.1 (loss of MCyR)	28.3 (alive)

ELN: European Leukemia Net, mo=months, MCyR: major cytogenetic response, EFS: event free survival, OS: overall survival, F: female, M: male

Table 4. Cox proportional hazard model multivariate analysis in 48 patients with CML

Co-variable		*Exp (B) Relative risk*	*EFS 95% CI*	*p-value*
Age (years)	≥60	2.741	0.647-11.601	0.171
Sokal risk score at diagnosis	High	0.638	0.196-2.079	0.456
Splenomegaly	Yes			0.958
Duplication	Group 2 (IMPDH1, FGFR1)	0.245	0.070-0.859	0.028

EFS: event-free survival, CI: confidence interval

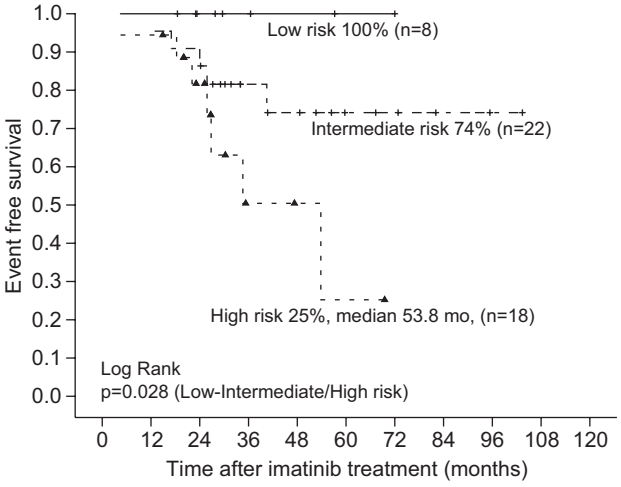

Figure 1. Kaplan-Meier plots on event-free survival time according to the type of Sokal risk score at diagnosis.

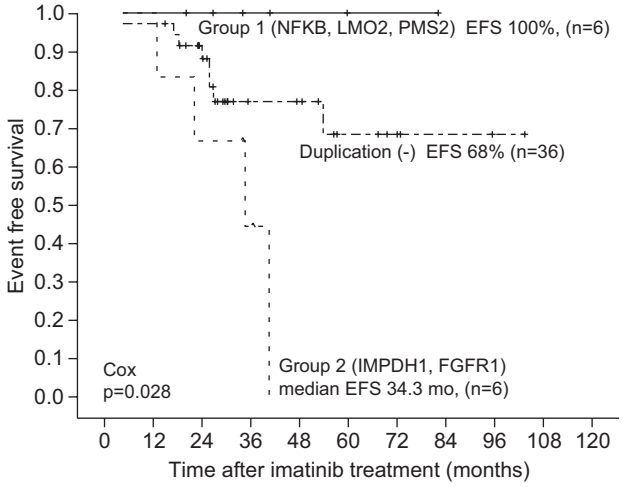

Figure 2. Kaplan-Meier plots on event-free survival (EFS) time according to the type of duplication.

Discussion

It is known that genes play various roles in carcinogenesis and tumor progression. New cancer-associated genes can be identified thanks to genome scans and the determination of mutations in the genomes of cancer patients. Depending on the roles they undertake, these genes are referred to as tumor suppressor genes, oncogenes or repair genes, and they play a role in many vital steps such as cell cycle, apoptosis, repair mecha-

nisms, transcription or signal transduction [13,14]. The MLPA is a method that is preferred because it is more sensitive and easily applicable than other methods performed using the short tandem repeat (STR) regions with PCR [8]. With this method, those chromosome regions where 67 genes were located were investigated in the DNA samples of 48 CML Ph+ patients. In this study, it was demonstrated that the genes we had selected for analysis also had originalities regarding some types of cancer and efficiency in the vital biological activities of the cell (such as apoptosis, cell cycle and DNA repair) in comparison with previous studies [4,9,15]. As it is stated, FGFR1, PMS2, IMPDH1, LMO2 and NFKB genes, in which duplication was found in our study, have been associated with many cellular functions and diseases in previous studies [9,16]. Of these genes, 2 (PMS2 and IMPDH1) are located on chromosome 7, 1 (FGFR1) on chromosome 8, 1 (NFKB) on chromosome 14 and 1 (LMO2) on chromosome 11 [13-16].

PMS2 gene is one of the genes that play a role in DNA mismatch repair (MER), a DNA repair mechanism. Other genes are MSH2, MLH1 and PMS1. PMS2 gene manifested itself with the deletion in 2 of 22 patients with a family history of HNPCC (hereditary nonpolyposis colon cancer) [17]. While heterozygote germline mutations were found in non-tumor tissues, somatic PMS2 mutation was found in the tumor tissue [18]. The mutations taking place in PMS2 gene, having a significant role in colorectal cancer, play a significant role in mismatch repair mechanism in cancer (also known as the Turcot syndrome) with autosomal recessive inheritance [19]. In a study conducted with some DNA mismatch repair genes, also including PMS2, it was advocated that the aberrations in mismatch repair develop in leukemia cell lines and acute lymphoid leukemia (ALL) more frequently than it was considered previously. Mutations and aberrations in PMS2 gene, that are very difficult to detect, have been identified in malignancies occurring both in children (with homozygote mutation or with duplication) and adults (with heterozygote mutation) [19-21]. The PMS2 gene duplication, which we detected in CML patients, was shown in this study for the first time; however, it was observed that there was no significant association with the analyzed clinical parameters.

NFKB is a transcription factor that controls numerous gene expressions responsible for cell growth and differentiation, regulation of apoptosis, and neoplastic transformation [22]. NFKB makes a significant contribution to resistance to lymphoma, and since it activates gene expression with respect to anti-apoptosis, it is considered that it might play an important role in tumor development and progression [23]. The NFKB pathway might either activate or inhibit cell death pathways in the pathological processes. Acute and severe NFKB activation in endotoxemia leads to increased cell membrane permeability, disseminated coagulation and extensive endothelial cell damage [24]. It was demonstrated that NFKB inhibition sensitized the bladder, breast and squamous cell cancer cell lines, besides fibrosarcoma, lymphoma and melanoma, against the cytotoxic effects of chemotherapy and radiotherapy [25,26]. The NFKB gene duplication, which we detected in CML patients, was shown in this study for the first time; however, it was observed that there was no significant association with the analyzed clinical parameters.

LMO2, the T-cell translocation gene, has an important role in erythropoiesis and T-cell leukemogenesis [14]. In the studies included in the international diagnosis index, it has been shown that LMO2, BCL6 and FN1 genes are associated with long-term survival and CCND2, SCYA3 and BCL2 are effective in short-term survival of B-cell lymphoma patients [15,27,28]. The LMO2 gene duplication, which we detected in CML patients, was shown in this study for the first time; however, it was observed that there was no significant association with the analyzed clinical parameters.

Some 67 genes, which were shown to be important for cancer etiopathogenesis, were analyzed in our study. The FGFR1 and IMPDH1 genes, detected to have significant associations with duplication and CML, are associated with many cellular functions and diseases [9,16]. Of these genes, IMPDH1 is located on chromosome 7 and FGFR1 on chromosome 8 [13]. The FGFR1 gene is one of several fibroblast growth factors that have crucial functions in cell division, growth and maturation, structure of blood vessels, recovery of injury, and embryonic development, and it appears with t(8;22) (p12;q11) in CML patients [29-31]. In the IMPDH1 gene, however, 2% of the cases in studies of mutation scanning performed with ADRP (autosomal dominant retinitis pigmentosa) carried mutations in this gene [9,13]. It was suggested that IMPDH1 mutations altered the single-stranded nucleic acid binding characteristic of the protein rather than affecting the enzyme activity itself [32,33]. Except Philadelphia positivity, different chromosomal variations were also found in CML and it was seen that these variations included those regions where the 2 genes- in which we detected duplication in our study- were located. For instance, some variations, in which chromosome 7 was included, were as follows: t(4;12;7;9;22)(q33?;q24;p13;q34;q11), t(7;14) (p22;q23), t(7;22)(p22;q11), t(7;9;22)(p22;q34;q111), and der(7)t(7;14) (p22;q13) [33-35]. Furthermore, t(7;8) (q32;q13) translocation, taking place between chromosomes 7 and 8, was identified in a case of acute basophilic leukemia (ABL) that has recently been identified

by the World Health Organization (WHO) and which is a different form of AML [29,36]. The best known variation of chromosome 8 is the observation of trisomy 8 in the blastic phase. Besides, t(8;21)(q22;q22), t(8;10;21), t(8;22) (p12;q11) and t(8;9) are other chromosomal variations that are identified in relation to chromosome 8 in CML [29-39]. In addition to the variations of these 2 chromosomes that were detected in previous studies [36,39] in CML, it is important that the duplication of IMPDH1 gene located on chromosome 7 and the duplication of FGFR1 gene located on chromosome 8, which were first detected in our study, were demonstrated. It was also observed that the presence of IMPDH1 and FGFR1 gene duplications in Ph+ CML patients particularly caused the 7-year median EFS duration to fall to around 34.3 months (Figures 1 and 2).

In conclusion, duplications in 5 genes in chromosomes 7, 8, 11 and 14, in addition to the Ph+ chromosome in CML patients taking imatinib were first detected in this study. It was determined that, especially the duplications of IMPDH1 and FGFR1 genes, located in chromosomes 7 and 8 and being in charge of signal transduction, had a negative effect on EFS. With this comprehensive original study that was conducted for the first time, it was put forward that -in addition to the Ph+ chromosome- especially chromosomes 7 and 8 must be investigated in more detail when determining disease prognosis and when selecting treatment alternatives in CML patients. According to the results of the present study, the association between CML and gene duplications can be further enlightened with similar studies with larger numbers of patient groups or with different populations.

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