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Research article

Study of Methylation Status in *TET2* Mutations in Iranian Breast Cancer Patient

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Abstract

TET2 enzymatic ally converts 5-methyl-cytosine to 5-hydroxymethyl-cytosine, possibly leading to loss of DNA methylation. Genetic mutations of TET2 gene were associated with leukemia, whereas TET1 down regulation has been shown to promote malignancy in breast cancer. To expand on this concept, we studied methylation status in TET2 gene in Breast Cancer (BC) samples. *TET2* Messene or nonsense mutations were detected in 53% (16/30) of patients. In contrast, only 1/30 patient had a mutation in *IDH1* or *IDH2*, and none of them had a mutation in *DNMT3A* in the sites most frequently mutated in Breast Cancer. Using bisulfate pyrosequencing, global methylation measured by the LINE-1 assay and DNA methylation levels of 10 promoter CpG islands frequently abnormal in myeloid leukemia were not different between *TET2* mutants and wild-type BC cases. This was also true for 9 out of 11 gene promoters reported by others as differentially methylated by *TET2* mutations. We found that two non-CpG island promoters, *AIM2* and *SP140*, were hyper ethylated in patients with mutant *TET2*. These were the only two gene promoters (out of 14,475 genes) previously found to be hyper ethylated in *TET2* mutant cases. However, total 5-methylcytosine levels in *TET2* mutant cases were significantly higher than *TET2* wild-type cases (median = 14.0% and 9.8%, respectively) (p = 0.016). Thus, *TET2* mutations affect global methylation in BC but most of the changes are likely to be outside gene promoters.

Keywords: TET2; DNA Methylation; Breast Cancer; Mutation; Promoter

Abbreviation

PCR: Polymerase Chain Reaction;

BC: Breast Cancer;

TET: Ten Eleven Translocation

Introduction

TET2 [*ten-eleven translocation* (TET) oncogene family member 2] is a tumor suppressor gene on chromosome 4q24 [1]. *TET2* mutations were first described in myeloproliferative neoplasms (MPN) [1-7]. As reported for *TET1* [8], *TET2* also converts 5-methyl-cytosine to 5-hydroxymethylcytosine [9] in embryonic stem cells, and thus mutations of *TET2* were proposed to contribute to leukemogenesis by altering epigenetic regulation of transcription through DNA methylation. Disrupting hematopoietic differentiation [10,11]. Furthermore, in murine models, *TET2* deficiency impairs hematopoietic differentiation with expansion of myeloid precursors [12,13]. The exact mechanism and the extent to which *TET2* mutations affect DNA methylation remain in question. In contrast, Figueroa et al. studied *TET2* mutant AMLs and identified a hyper methylation phenotype, including 129 differentially methylated regions [11]. These studies were conducted using microarray-based screening methods for DNA methylation analysis, which might have false positive and negative findings. To examine this issue in more detail, we used bisulfite pyrosequencing, which is one of the most reliable ways to analyze DNA methylation for in-

dividual genes, and probed the DNA methylation status of 21 promoters of interest as well as global DNA methylation levels in a cohort of 30 BC patients. Our data suggest that effects of *TET2* mutations on DNA methylation are primarily outside gene promoters.

Patients and Methods

Patients

We analyzed peripheral blood samples prior to treatment from 30 patients with BC referred to The Loqman Hakim Hospital Cancer Center in Tehran. All patients gave informed consent for the collection of residual tissues as per institutional guidelines.

Mutation analysis

For *TET2* gene analysis, polymerase chain reaction (PCR) and direct sequencing of exon 3– 11 was performed starting from 20 ng of genomic DNA, as previously described [1]. PCR amplicons were sequenced by Beckman Coulter Genomics (Danvers, MA). All *TET2* mutations were scored on both strands. Sequence traces were analyzed with SeqMan Pro (DNASTAR, Inc., WI) and reviewed visually. Previously annotated single nucleotide polymorphisms in the HapMap database (www.hapmap.org) were discarded. SIFT software [26] was used to determine the probability that a particular amino acid substitution is tolerated.

Quantitative DNA methylation analyses by bisulfite pyrosequencing

We used bisulfite pyrosequencing to quantitatively assess DNA methylation [30] for 10 promoter CpG islands frequently abnormal in MDS [*ER (ESR1), NOR1 (OSCP1), p15 (CDKN2B), NPM2, ECAD (CDH1), CDH13, OLIG2, PGRB, PGRA* and *RIL (PDLIM4)*], [15] and 11 promoter regions of genes reported by others to be differentially methylated by *TET2* mutations (hypomethylated: *C9orf16*, *PSMD6, LRRC32, TMEM34, FSD1NL*; hypermethylated: *AIM2* and *SP140* from Ko et al. and *ACOX3, SLC39A14, ZNF662* and *DNM3* from Figueroa et al.). Long interspersed nuclear element-1 (LINE-1) was also analyzed to measure global repeat element methylation. We tested samples for which a sufficient amount of DNA was available for bisulfite treatment. The number of patients with successful results (mostly >90% success rate) varied slightly for each gene.

Measurement of 5-methyl-cytosine levels by mass spectrometry

DNA hydrolysis was performed as previously described by Song et al. with minor modifications [11,31]. Briefly, one microgram of genomic DNA was first denatured by heating at 100°C. Five units of Nuclease P1, were added and the mixture incubated at 45°C for 1 h. A 1/10 volume of 1 M ammonium bicarbonate and 0.002 units of venom phosphodiesterase 1 were added to the mixture and the incubation continued for 2 h at 37°C. Next, 0.5 units of alkaline phosphatase were added, and the mixture incubated for 1 h at 37°C. Before injection into the Zorbax XDB-C18 2.1 mm x 50 mm column (1.8 µm particle size), the reactions were diluted 10-fold to dilute out the salts and the enzymes. Samples were run on an Agilent 1200 Series liquid chromatography machine in tandem with the Agilent 6410 Triple Quad Mass Spectrometer. LC separation was performed at a flow rate of 220 µL/min. Quantification was done using a LC-ESI-MS/MS system in the multiple reaction monitoring (MRM) mode. We measured 5-methyl-cytosine levels in genomic DNA of *TET2* mutant and wild-type cases where sufficient amount of samples are available in quantity for mass spectrometry (12 mutant and 7 wild-type cases).

Statistical Analysis

Statistical analyses were performed using PRISM (GraphPad Software, Inc., CA). Differences in clinical characteristics of patients with or without TET2 mutations were assessed using the Fisher's exact test, the Mann-Whitney or log-rank analysis. We used Kaplan-Meier tests to calculate and generate survival curves and used the log-rank test to determine significance between the group of TET2 mutant and wild-type. We used the Mann Whitney test to compare continuous variables of DNA methylation and 5-methyl-cytosine levels between TET2 mutant and wild-type cases. All p values were two-tailed and the threshold of statistical significance was p less than 0.05 followed by Bonferroni's correction when multiple analyses were performed.

Results

TET2 mutation status in BC

We analyzed the nature and frequency of somatic mutations affecting the *TET2* coding sequence (exons 3–11) in a cohort of 30 patients with BC according to WHO criteria. *TET2* missense or nonsense mutations were detected in 16 out of 30 (53%) patients. Ten patients had a single heterozygous mutation, two had a biallelic or homozygous mutations, three had two mutations, and one patient had three distinct mutations. Altogether, 21 mutations were identified, including 7 missense, 7 nonsense and 7 frame shift mutations. Detailed mutation information is shown in Table 1. Mutations were observed in exon 3 (8 events), exon 5 (1 event), exon 6 (3 events), exon 7 (1 event), exon 9 (1 event), exon 10 (2 events) and exon 11 (5 events). Only 5 out of 21 identified mutations have been reported previously. Six out of seven identified missense mutations were predicted to affect protein function by using SIFT software. We identified *IDH2* R140Q mutation in 1 out of 30 BC patients (3%). Mutation at the R882 residue in *DNMT3A* was found in 21 of the 30 BC patients.. We compared the overall and progression free survival in patients with vs. without mutations and observed no significant differences.

Patient	Nucleotide change	Amino acid change
MT1	c.1623C > T	Q255X
MT2	Del c.3022_3023 (CA); Ins 4636 (GCTCA)	H721FS; T1259FS
MT3	c.2820C > T; c.4559C > A	Q654X; W1233X
MT4	c.4500C > T; c.5850C > T	R1214W[†]; Q1664X
MT5	c.6508C > T	T1883I
MT6	c.5163C > T	Q1435X[*]
MT7	Ins c.2617 (T); c.3869G > A; c.5469C > T	L586FS; W1003X; Q1537X[†]
MT8	c.4998C > T	H1380Y
MT9	Del c.3442 (A)	N861FS
MT10	Del c.5521_5524 (CAGA)	T1554FS[†]
MT11	c.4435G > T	G1192V
MT12	Ins c.2519 (G)	V553FS
MT13	c.6012G > T	V1718L[†,§]
MT14	c.4753G > A	C1298Y
MT15	Del c.2653 (A)	N598FS
MT16	c.5109G > T	V1417F[*,†]

[*]Biallelic/homozygous mutations;
[†]Previously reported;
[§]Predicted to be tolerated.

Table 1. *TET2* mutation status

Comparison of DNA methylation levels between *TET2* mutant and wild-type cases.

Next, we performed bisulfite pyrosequencing to compare DNA methylation status between patients with mutant vs. wild-type *TET2* genes (Table 2). Bisulfite pyrosequencing is a highly quantitative and reliable method for methylation analysis of individual CpG sites. We compared *TET2* mutant to *TET2* wild-type cases to distinguish the effects of *TET2* on methylation from the effects of BC transformation. First, we studied DNA methylation levels of 10 promoter CpG islands frequently abnormal in MDS [15] since these genes are assumed to be most likely to show abnormal DNA methylation levels in their promoters when the DNA methylation machinery is altered.

Discussion

In this cohort of 30 BC patients, we found that missense or nonsense mutations of *TET2* were detected in 16 out of 30 (53%) patients. Mutations were found to be distributed broadly from exon 3 to exon 11. Furthermore, only 5 out of 21 mutations were the same as previously reported, confirming the marked heterogeneity in mutational status. Overall, in addition to the frequency of mutations, the characteristics of the mutations in this study are in good agreement with what has been reported so far. Missense mutations and frame shift mutations are mainly found in exon 3 of *TET2,* whereas point mutations are found in exons 4 to 11.

Although these analyses revealed that *TET2* does not have mutation "hot spot(s)" as seen for *IDH1/2* and *DNMT3A* in MDS, some locations in *TET2* were found to have high frequencies of mutations. We also confirmed that 21 of 30 BC patients had mutation at the R882 residue in *DNMT3A*. We could not find significant differences in overall and progression free survival between *TET2* mutant and wild-type cases in this cohort. However, correlation of TET2 mutation and survival is still in question; reported by different studies as superior in MDS [19] and BC. Larger studies will be needed to confirm the effect of *TET2* mutations on survival.

Bisulfite pyrosequencing is one of the most reliable ways to analyze DNA methylation for individual genes, and we find that only two genes, *AIM2* and *SP140*, were hypermethylated in patients with mutant *TET2* compared with wild-type *TET2.* These genes are the only two genes found to be hypermethylated in a previous report that studied 14,475 genes [10]. Recently, *AIM2* was reported to have a putative role in reduction of cell proliferation by cell cycle arrest [20]; therefore, methylation of the promoter might provide a growth advantage to cancer cells.

		TET2 mutant (n = 16)			*TET2* wild type (n = 14)			Normal peripheral blood (n = 5)			p value
			Range			Range			Range		(*TET2* mutant vs. wild type)
Gene	CpG island	Median (%)	Min (%)	Max (%)	Median (%)	Min (%)	Max (%)	Median (%)	Min (%)	Max (%)	
ER (ESR1)	Y	3	1	36	5	0	23	4	1	5	0.69
NOR1 (OSCP1)	Y	2	0	24	2	0	18	3	1	4	0.73
p15 (CDKN2B)	Y	7	0	29	8	2	15	3	1	7	0.76
NPM2	Y	6	1	17	7	0	14	3	2	4	0.68
ECAD (CDH1)	Y	5	2	31	10	2	39	7	6	9	0.25
CDH13	Y	14	0	29	16	3	43	6	4	8	0.27
OLIG2	Y	10	3	37	14	4	35	6	3	8	0.32
PGRB	Y	28	6	46	16	5	54	6	1	11	0.04
PGRA	Y	15	0	40	12	1	34	5	2	6	0.71
RIL (PDLIM4)	Y	19	8	68	28	5	68	21	12	33	0.56
C9orf16	Y	5	1	34	4	0	14	16	0	27	0.72
PSMD6	N	44	18	80	41	0	81	51	48	58	0.42
LRRC32	N	13	6	50	23	7	63	40	29	47	0.14
TMEM34	Y	22	2	42	13	1	68	16	8	22	0.33

Table 2. DNA methylation levels

Methylation of *SP140* might have an effect on differentiation to specific lineages. Overall, we find rare promoter methylation differences in *TET2* mutant cases, but hypermethylation of *AIM2* and *SP140* may be useful biomarkers of *TET2* mutations in BC.

TET2 mutation has been shown to lead to inefficient conversion of 5-methyl-cytosine to 5hydroxymethyl-cytosine. Consistent with this, we found that 5-methyl-cytosine levels of *TET2* mutant cases are higher than *TET2* wild-type cases. However, this does not seem to translate to increased promoter methylation, with *AIM2* and *SP140* being notable exceptions. While we did not study the whole genome to be completely confident of this fact, we did investigate the most frequently hypermethylated genes in MDS, and others studied genomewide methylation with similar findings (hypermethylation of only two out of 14,475 genes). We could not confirm hypomethylation in *TET2* mutant BC cases. Given the above, our findings suggest that the total methylation level increase in *TET2* mutant cases is mostly outside CpG islands and promoters examined so far.

There are several possible explanations for the findings in this study about the impact of *TET2* mutations on promoter methylation. First, different *TET2* mutations might affect DNA methylation in divergent ways; however, most of the mutations found in this report are predicted to negatively affect protein function. We found no difference in DNA methylation in patients with homozygous, biallelic or frame-shift mutations. To support this, the only two differentially methylated genes in this study, *AIM2* and *SP140*. It is also possible that the effect on promoter DNA methylation of *TET2* is not global but very restricted to a few genes such as *AIM2* and *SP140*. However, the TET1 protein has been found to be enriched at most CpG-rich sequences [23,24] and there is no mechanism to explain selectivity. Because the promoters of *AIM2* and *SP140* are not in CpG islands, the observed effect on DNA methylation could be secondary to other effects of *TET2* on gene expression. Indeed, TET1 protein has been found to affect gene expression independent of DNA methylation [23]. Altogether our data suggest that *TET2* mutations have effects on global DNA methylation, but we have not been able to detect major effects on promoter methylation (with the limitations previously discussed). It appears likely that *TET2* mutations affect DNA methylation in other regions such as gene bodies or intergenic areas. Larger and genome wide studies will be needed to confirm the precise relationship between *TET2* mutations and DNA methylation.

Conclusions

In conclusion, we have shown that epigenetic markers that TET2 gene is one of them, are promising biomarkers for breast cancer. The results presented in this thesis do not unambiguously indicate that altered epigenetic regulation is responsible for the unique methylation pattern observed in samples from patients with Breast cancer. Further research is needed to fully understand the biology of Breast cancer. This study highlights the potential for TET2 methylation to be an informative prognostic biomarker for breast cancer survival and sets the scene for a more comprehensive investigation of the molecular basis of this phenomenon.

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