Research Article

Death associated protein kinase-1 gene methylation pattern in some leukemic patients attending Zagazig University hospitals: is it a clue?

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Received: 30 July 2016
Accepted: 04 August 2016

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ABSTRACT

Background: Leukemia is a type of cancer arising from white blood cells (WBCs) and resulting from malignant transformation of different types of white blood cell precursors. The objective was to study the DAPK-1 gene methylation pattern in leukemic patients and to through some light on its possible role as a risk factor for leukemia.

Methods: Forty-one patients diagnosed as leukemic patients and 41 age-matched healthy unrelated volunteers taken as a control group. The analysis of aberrant promoter DAPK1 gene methylation was done by specific polymerase chain reaction.

Results: The results of the present study showed that there was a significant association of methylated DAPK-1 promoter area among leukemic group than in control group Chi-square (X²) was 21.98, or value patients was 10.46 and there was a significant association when compared with the control group (p <0.001). And there was no significant association when compared according to gender Chi-square (X²) was 0.43 and (p=0.51). Our results revealed in the AML group DAPK-1 promoter area were methylated with percentage of 73.9%. or value for AML patients was 13.76 and there was a significant association when compared with the control group (p <0.001), in the ALL group 4 patients had methylated DAPK-1 promoter area with percentage of 57.1% or value for all patients was 6.47 and there was a significant association when compared with the control group (p=0.03) and in the CLL group 7 patients had methylated DAPK-1 promoter area with percentage of 63.6%. OR value for CCL patients was 8.5 and there was a significant association when compared with the control group (p=0.004). On the contrary, we didn’t observe any significant associations between DAPK-1 promoter area methylation and the type of leukemia (p = 0.65).

Conclusions: These results suggested that DAPK1 promoter methylation might play a significant role in the pathogenesis of different types of leukemia. And the DAPK1 promoter methylation has a predictive value in the prediction of leukemia occurrence.

Keywords: Leukemia, Death associated protein kinase, Pattern of methylation, Methylation specific polymerase chain reaction (MSP-PCR)

INTRODUCTION

Leukemia is a type of cancer arising from white blood cells (WBCs) and resulting from malignant transformation of different types of white blood cell precursors. Leukemic cells grow and divide uncontrollably, displacing healthy blood cells. This can lead to serious problems such as anemia, bleeding, and infection.1-3

DNA methylation is a process by which methyl groups are added to DNA. Methylation modifies the function of the DNA. When located in a gene promoter, DNA methylation typically acts to repress gene transcription.4
Unmethylated CpGs are often grouped in clusters called CpG islands, which are present in the 5' regulatory regions of many genes. In many disease processes, such as cancer, gene promoter CpG islands acquire abnormal hypermethylation, which results in transcriptional silencing that can be inherited by daughter cells following cell division.5

Death-associated protein kinase 1 is a positive mediator of gamma-interferon induced programmed cell death. DAPK1 encodes a structurally unique 160-kD calmodulin dependent serine-threonine kinase that carries 8 ankyrin repeats and 2 putative P-loop consensus sites. It is a tumor suppressor candidate.6

Promoter methylation of DAPK inactivates its transformation suppression function. It was shown to be inactivated by methylation in a variety of human tumors, including B cell lymphoma, small cell lung cancer, multiple myeloma, gastrointestinal malignancies.7,9

Gonzalez et al. showed 14% DAPK1 aberrant methylation in neuroblastic tumours.10 Sung et al. detected 60.9% promoter DAPK1 methylation in B-cell lymphoma in Korean population.11 A study by Eknek et al. observed the frequency of DAPK 1 methylation in pediatric AML and adult AML by 70% and 55% respectively.12 67% methylation was detected in multiple myeloma by Margaret HL et al.13 Also 23.20% promoter DAPK1 methylation was detected in childhood acute lymphoblastic leukemia.14 In South Korean population DAPK1 promoter had 94.3% methylation in ocular adnexal lymphoma.15 In different haematological malignancies aberrant 28% DAPK1 methylation was reported.16

Promoter DAPK1 methylation contribute to the genetic influence on leukemia. On the basis of the central role of the promoter DAPK1 methylation in leukemia, it was hypothesized that DAPK-1 gene methylation pattern is a risk factor for leukemia.

METHODS

This study was Carried Out at the Medical Biochemistry Department and Internal medicine department, Faculty of medicine, Zagazig University. Forty-one patients diagnosed as leukemic patients and 41 age-matched healthy unrelated volunteers taken as a control group. None of the cases had past history of malignancy anywhere or family history, any other disease that may interfere with the study parameters or were deteriorated cases.

The cases included 41 patients diagnosed as leukemic patients, 21 males and 20 females and there ages ranged from 22 to 62 years with a mean±SD of 39.5±10.9 years. They were diagnosed as leukemic patients in the Internal Medical Department in accordance to clinical and laboratory data. This group included 23 AML (acute myeloid leukemia) patients, 14 males and 9 females, there ages ranged from 24 to 59 years with a mean±SD of 37.4±9.9 years. The initial clinical presentation of AML largely depends on the extent of the leukemic infiltration of the bone marrow and extramedullary sites. AML classification is based on lineage-associated phenotype (undifferentiated, myeloid, monoblastic, erythroblastic or megakaryoblastic).

Also included 7 ALL (acute lymphocytic leukemia) patients, 4 males and 3 females, there ages ranged from 23-57 years with a mean±SD of 35.3±7.3. Clinical signs were fever, pallor, fatigue, bruises, enlargement of liver, spleen and lymph nodes and pain (e.g. bone pain). In most patients, white blood cell counts showed anemia, thrombocytopenia and granulocytopenia with or without concomitant leukocytosis. Eleven patients diagnosed with CLL (chronic lymphocytic leukemia) patients, 4 males and 7 females, there ages ranged from (29-60) years with a mean±SD of 36.2±9.7. The diagnosis of different leukemic type is made by cytomorphological and cytochemical examination of a bone marrow aspirate and/or peripheral blood leukemic cells.17,18 All the patients and healthy individuals gave their written consent before blood sample collection. In addition to a full history and clinical examination, routine investigations including X-ray for chest, liver function, kidney function, serum uric acid, fasting blood sugar analysis was performed in all cases to exclude factors that could influence measurements.

Collection of blood samples

Six ml of blood sample was taken from every participant under complete aseptic condition. The whole blood was collected in sterile EDTA (solute form) containing tubes and kept frozen at -20°C till analysis.

DNA extraction

DNA was isolated using the TIANamp genomic DNA kit purchased from Tiangen.

Bisulfite treatment

We used Epi Tect Fast DNA bisulfite conversion kit (Qiagen, Germany). We prepared the bisulfite reactions in 200 µl PCR tubes. (10 µl of DNA, 10 µl of RNase-free water, 85 µl of bisulfite solution and 35 µl of DNA protect buffer ) to reach a total volume of 140 µl. The PCR tubes were putted in a thermal cycler PTC-100 (MJ Research, Inc. Watertown, Mass. USA). The thermal cycling conditions as follows (denaturation at 95°C for 5 min, incubation at 60°C for 10 min, denaturation at 95°C for 5 min, Incubation at 60°C for 10 min. and then hold at 20°C.

Original sequence

Unmethylated DNA N-C-G-N-C-G-N-C-G-N
Methylated DNA N-C-G-N-C-G-N-C-G-N

**After bisulfite treatment**

N-U-G-N-U-G-N-U-G-N

N-C-G-N-C-G-N-C-G-N

**Methylation-specific polymerase chain reaction**

The amplification was carried out using thermal cycler PTC-100 machine (MJ Research, Inc, Watertown, Mass. USA) to perform three phases of PCR reaction (denaturation→annealing→extension of primers of DAPK-1). The methylated primers of DAPK -1 were 5’-GGA TAG TCG GAT CGA GTT AAC GTC-3’ (sense) and 5’-CCC TCC CAA ACG CCG A-3’ (antisense) ; the unmethylated primers of DAPK -1 were 5’-GGA GGA TAG TGT GAT TGA GTT AAT GTT (sense) and 5’-CAA ATC CCT CCC AAA CAC CAA-3’ (anti- sense).

PCR was performed in a final volume of 25 μL containing 5.5 μL of H₂O, 5 μL (2 μg) of bisulfite-treated DNA by PCR Master Mix kit, 1 μL of each primer (1 μM), and 12.5 μL of 2X PCR Master mix solution (Qiagen , Germany) (12.5 μL). The PCR conditions as follows: a denaturing step at 94°C for 2 min is followed by 35 cycles of [94°C for 20 sec, the annealing temperature at 60°C for 20 sec, and finally extension at 72°C for 30 sec], then final extension at 72°C for 2 minutes. Ten microliters of each PCR were directly loaded onto 2.5% agarose gels, stained with (5mg/ml) ethidium bromide, and visualized under UV illumination. The methylated base pair length was 103 bp while the unmethylated was 98 bp.

Ten microliters of each PCR were directly loaded onto 2.5% agarose gels, stained with (5 mg/ml) ethidium bromide, and visualized under UV illumination. Statistical analysis was conducted using version 11 of the statistical package SPSS for Windows. A p-value of <0.05 was considered statistically significant.

**RESULTS**

**Methylation frequency**

Twenty eight cases were methylated DAPK-1 promoter area among leukemic patients with percentage of 68.3% from which 16 males and 12 females. There was a significant association of methylated DAPK-1 promoter area among leukemic group than in control group chi-square (X²) was 21.98, or value patients was 10.46, 95% CI was (3.31-34.6) and there was a significant association when compared with the control group (p=0.001). And there was no significant association when compared according to gender Chi-square (X²) was 0.43 and (p=0.51).

**Table 1: Frequency of aberrant DAPK-1 methylation by PCR.**

<table>
<thead>
<tr>
<th></th>
<th>Control no. percent</th>
<th>Cases no. percent</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylated</td>
<td>7 (17.1%)</td>
<td>28 (68.3%)</td>
<td>10.46</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>34 (82.9%)</td>
<td>13 (31.7%)</td>
<td>(3.31-34.6)</td>
</tr>
</tbody>
</table>

**Table 2: Frequency of aberrant DAPK-1 methylation by PCR according to gender.**

<table>
<thead>
<tr>
<th></th>
<th>Male no. (n=22)</th>
<th>Female no. (n=19)</th>
<th>(X²) P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylated DAPK-1</td>
<td>16 (72.7%)</td>
<td>12 (63.2%)</td>
<td>0.43 (0.51)</td>
</tr>
<tr>
<td>Unmethylated DAPK-1</td>
<td>6 (27.3%)</td>
<td>7 (36.8%)</td>
<td></td>
</tr>
</tbody>
</table>

Seventeen patients in the AML group DAPK-1 promoter area was methylated with percentage of 73.9% from which 9 males and 8 females or value for AML patients was 13.76, 95% CI was (3.47-58.99) and there was a significant association when compared with the control group (p <0.001). In the all group 4 patients had methylated DAPK-1 promoter area with percentage of 57.1% from which 2 males and 2 females or value for all patients was 6.47, 95% CI was (1.178-35.58) and there was a significant association when compared with the control group (p=0.03). And in the CLL group 7 patients had methylated DAPK-1 promoter area with percentage of 63.6% from which 3 males and 4 females or value for CLL patients was 8.5, 95% CI was (1.94-37.09) and there was a significant association when compared with the control group (p=0.004).

While in the control group 7 individuals only had methylated DAPK-1 promoter area with percentage of 17.1% from which 3 males and 4 females.

**Table 3: Comparison between type of leukemia and control groups as regard methylation.**

<table>
<thead>
<tr>
<th></th>
<th>All no. percent</th>
<th>CI no. percent</th>
<th>AML no. percent</th>
<th>(X²) P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylated</td>
<td>4 (57.1%)</td>
<td>7 (63.6%)</td>
<td>17 (73.9%)</td>
<td>0.85 (0.65)</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>3 (42.9%)</td>
<td>4 (36.4%)</td>
<td>6 (26.1%)</td>
<td></td>
</tr>
</tbody>
</table>

The DAPK-1 promoter area methylation of cases group was no significant association when compared according to type of leukemia (chi-square (X²) was 0.14 and (p=0.93).
Table 4: Frequency of aberrant DAPK-1 methylation by PCR according to type of leukemia.

<table>
<thead>
<tr>
<th></th>
<th>Unmethy whole no. percentage</th>
<th>Methyl at ion no. percentage</th>
<th>or 95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>34</td>
<td>7</td>
<td>6.47</td>
<td>0.0316</td>
</tr>
<tr>
<td>ALL</td>
<td>33</td>
<td>4</td>
<td>1.178</td>
<td></td>
</tr>
<tr>
<td></td>
<td>42.9%</td>
<td>57.1%</td>
<td>35.58</td>
<td></td>
</tr>
<tr>
<td>CLL</td>
<td>34</td>
<td>7</td>
<td>8.5</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>82.9%</td>
<td>17.1%</td>
<td>1.94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>7</td>
<td>37.09</td>
<td></td>
</tr>
<tr>
<td>36.4%</td>
<td>63.6%</td>
<td>37.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>34</td>
<td>7</td>
<td>13.76</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AML</td>
<td>34</td>
<td>7</td>
<td>34.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>82.9%</td>
<td>17.1%</td>
<td>58.99</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>26.1%</td>
<td>73.9%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The cellular level of DAPK can be regulated manifold. On the transcriptional level promoter hypermethylation has been described that strongly correlates with DAPK protein loss. The promoter of DAPK has a high density of CpG islands and motifs for a number of transcription factors are located within these regions such as for NFκB, e2f1 or AP1 DAPK suppresses oncogene-induced transformation induced by p19ARF-dependent activation of p53. It acts as helper of tumor suppressor genes. Methylation profile of the promoter CpG islands of DAPK is a target for cancer. Promotor methylation of DAPK inactivates its transformation suppression function. It was shown to be inactivated by methylation in a variety of human tumors, including B cell lymphoma, small cell lung cancer, multiple myeloma, gastrointestinal malignancies. Promoter DAPK1 methylation contribute to the genetic influence on leukemia. On the basis of the central role of the promoter DAPK1 methylation in leukemia, it was hypothesized that DAPK-1 gene methylation pattern is a risk factor for leukemia.

Therefore, we explored the possible role of methylation pattern of DAPK1 promoter in Egyptians as risk factor of leukemia and its subtypes.

There was a significant association of methylated DAPK-1 promoter area among leukemic group than in control group Chi-square ($X^2$) was 21.98, or value patients was 10.46, 95% CI was (3.31-34.6) and there was a significant association when compared with the control group (p < 0.001). And there was no significant association when compared according to gender Chi-square ($X^2$) was 0.43 and p=0.51. In the AML group DAPK-1 promoter area was methylated with percentage of 73.9%. or value for AML patients was 13.76, 95% CI was (3.47-58.99) and there was a significant association when compared with the control group (p <0.001).

Similar to our finding, Wang et al found that DAPK1 methylation is implicated in the disruption of the tumor suppressor network. Copland et al assumed that the critical nature of DAPK in regulating apoptosis under normal conditions is highlighted by the findings that malignant cells employ multiple mechanisms to abolish DAPK function, thus creating pro-survival conditions and promoting the initiation of cancer. Chım CS et al found that cancer cells are associated with global hypomethylation but with focal hypermethylation of specific gene promoters organized as CpG island. Celik et al found that the incidence of DAPK1 methylation was significantly higher in the resistant patients compared with the non-resistant and there was no DAPK1 methylation in any of the healthy controls.

Wei Q-X et al identified B-lymphoid malignancy related cell line models harboring allelic imbalance and found that allele-specific methylation in DAPK1 is associated with ASE.

DISCUSSION

Leukemia is a type of cancer arising from white blood cells (WBCs) and resulting from malignant transformation of different types of white blood cell precursors. Leukemic cells grow and divide uncontrollably, displacing healthy blood cells. This can lead to serious problems such as anemia, bleeding, and infection. Cancer is caused by the accumulation of genetic and epigenetic mutations in genes that normally play a role in the regulation of cell proliferation, thus leading to uncontrolled cell growth. Cells acquire mutations in these genes as a result of spontaneous and environmentally-induced DNA damage. Those cells with mutations that promote a growth and survival advantage over normal cells are selected for through a Darwinian process, leading to the evolution of a tumor. DNA methylation is a process by which methyl groups are added to DNA. Methylation modifies the function of the DNA. When located in a gene promoter, DNA methylation typically acts to repress gene transcription. DNA methylation is essential for normal development and is associated with a number of key processes including genomic imprinting, X-chromosome inactivation, repression of repetitive elements, aging and carcinogenesis.

Unmethylated CpGs are often grouped in clusters called CpG islands, which are present in the 5’ regulatory regions of many genes. In many disease processes, such as cancer, gene promoter CpG islands acquire abnormal hypermethylation, which results in transcriptional silencing that can be inherited by daughter cells following cell division. Death-associated protein kinase 1 is a positive mediator of gamma-interferon induced programmed cell death. DAPK1 encodes a structurally unique 160-kD calmodulin dependent serine-threonine kinase that carries 8 ankyrin repeats and 2 putative P-loop consensus sites. It is a tumor suppressor candidate.

The promoter of DAPK has a high density of CpG islands and motifs for a number of transcription factors are located within these regions such as for NFκB, e2f1 or AP1 DAPK suppresses oncogene-induced transformation induced by p19ARF-dependent activation of p53. It acts as helper of tumor suppressor genes. Methylation profile of the promoter CpG islands of DAPK is a target for cancer. Promotor methylation of DAPK inactivates its transformation suppression function. It was shown to be inactivated by methylation in a variety of human tumors, including B cell lymphoma, small cell lung cancer, multiple myeloma, gastrointestinal malignancies. Promoter DAPK1 methylation contribute to the genetic influence on leukemia. On the basis of the central role of the promoter DAPK1 methylation in leukemia, it was hypothesized that DAPK-1 gene methylation pattern is a risk factor for leukemia.

Therefore, we explored the possible role of methylation pattern of DAPK1 promoter in Egyptians as risk factor of leukemia and its subtypes.
However, other studies doubt on the role of methylation status of DAPK1 gene as an important indicator of leukemia. Claus, R et al found that there was almost complete absence of elevated DNA methylation among AML and MDS patients.26 This difference could be explained by different population who were included in the study or to the different age or due to the severity of the disease.

Among the ALL patients group 4 patients had methylated DAPK-1 promoter area with percentage of 57.1 %. OR value for ALL patients was 6.47, 95% CI was (1.178-35.58) and there was a significant association when compared with the control group (p = 0.03). And in the CLL group 7 patients had methylated DAPK-1 promoter area with percentage of 63.6 %. OR value for CCL patients was 8.5, 95% CI was (1.94-37.09) and there was a significant association when compared with the control group (p = 0.004).

In the same line of our data, Raval A et al, showed that loss or reduced expression of death-associated protein kinase 1 (DAPK1) underlies cases of heritable predisposition to CLL and the majority of sporadic CLL.23 Uehara E et al suggested that hypermethylation of cell cycle control genes play a significant role in the progression of CML.30 Mir R et al concluded that methylation status of DAPK1 gene is associated with advanced phase of CML and may be related to disease progression in chronic myeloid leukemia.34

Putting these findings together, indicate that status of DAPK1 methylation is likely to play a critical role in different types and stages of leukemia. These results suggested that DAPK1 promoter methylation might play a significant role in the pathogenesis of different types of leukemia.

The DAPK-1 promoter area methylation of cases group was no significant association when compared according to type of leukemia (Chi-square (X2) was 0.85 & (p = 0.65). Future work will be required to identify gene-gene and gene-environment interactions on a genome-wide level, with the aim of fully elucidating the genetic risk factors for different types of leukemia, understanding the pathogenesis of these diseases.

CONCLUSION

These results suggested that DAPK1 promoter methylation might be useful genetic biomarkers of leukemia and their subtypes in Egyptian patients.

ACKNOWLEDGMENTS

The authors would like to acknowledge scientific and medical research center “ZSMRC” of Zagazig Faculty of medicine for their support.

Funding: No funding sources
Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

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Cite this article as: Sarhan WM, Abou ElMagd YE, EL-Shal AS, Abd El Halim AF. Death associated protein kinase-1 gene methylation pattern in some leukemic patients attending Zagazig University hospitals: is it a clue? Int J Res Med Sci 2016;4: 3679-84.