

## RESEARCH ARTICLE

# Promyelocytic Leukemia (PML) Gene Mutations may not Contribute to Gastric Adenocarcinoma Development

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### Abstract

Gastric cancer is the second most common cause of cancer death worldwide. Environmental as well as genetic factors have been shown to be involved in its genesis. Among genetic factors, loss of function of a tumor suppressive gene named promyelocytic leukemia (PML) has been demonstrated in gastric cancer. In order to cast light in the mechanism by which PML protein is under-expressed in gastric cancer cells, we analyzed all exons and intron-exon boundaries of PML gene in 50 formalin-fixed paraffin-embedded tissue blocks from gastric carcinoma tumors by means of PCR-SSCP and CSGE, with direct sequencing of abnormally shifted bands. We found a novel sequence variant of unknown significance localized in intron 5 in 3 samples (c.1398+84delA). We did not detect any deleterious mutations of the PML gene. This study shows that PML mutations may not contribute to gastric adenocarcinoma development. Post-translational modifications or protein degradation might be mechanisms by which PML is not expressed in gastric tumors.

**Keywords:** PML - mutation - gastric cancer

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### Introduction

Despite the dramatic decrease in the incidence and mortality of gastric cancer during the last decades, it is still the second most common cause of cancer death worldwide (Nagini, 2012). Iran, as one of the high-risk areas for gastric cancer, has experienced an increase in gastric cancer incidence during recent years. Although this increase might be partly because of improvement in cancer registry systems, changes in life style and dietary pattern are considered as important factors contributing in the growing trend in gastric cancer incidence (Haidari et al., 2012). In Iran gastric cancer is the most common cancer in male and the third cancer in female (Babaei et al., 2010). Gastric adenocarcinoma can be classified as intestinal and diffuse types, or differentiated and undifferentiated types according to the Lauren and the Nakamura classification systems respectively. Intestinal-type adenocarcinoma is generally considered to be equivalent to differentiated adenocarcinoma, as is diffuse-type and undifferentiated adenocarcinoma. However, differentiated adenocarcinoma of the stomach is classified into gastric or intestinal phenotypes based on mucus expression (Namikawa and Hanazaki, 2010). Infection with *Helicobacter pylori* as a gastric pathogen has been shown to significantly increase the risk of developing gastric cancer (Wroblewski et al., 2010; Karami et al., 2013). In addition to environmental

factors, genetic factors contribute in this malignancy. In brief, activation of oncogenes, loss of tumor suppressor genes, and mutation of genes involved in DNA repair have been detected in the process of gastric carcinogenesis (Kyrlagkitsis and Karamanolis, 2003).

The promyelocytic leukemia (PML) gene is a gene known to be a tumor suppressor. It was firstly discovered in acute promyelocytic leukemia (APL) in which a t(15; 17) chromosomal translocation fused it to the retinoic acid receptor alpha (RAR $\alpha$ ) (Imani-Saber and Ghafouri-Fard, 2014). PML is concentrated in a distinct sub-nuclear compartment named the PML nuclear body (NB) (Zhong et al., 2000). PML co-localizes with more than 30 different proteins within the NBs, including some important tumor suppressor genes such as p53 and pRb (Salomoni and Pandolfi, 2002). PML protein expression has been shown to be reduced or abolished in gastric cancer, and its loss of expression is associated with more extensive lymphatic invasion, higher pTNM staging, and adverse prognosis (Lee et al., 2007). In addition, Epstein-Barr virus (EBV) infection, which is detected in the tissue of about 10% of gastric carcinoma cases (Takada, 2000; Campos et al., 2006), has been shown to lead to loss of PML-NBs, resulting in impaired responses to DNA damage and promotion of cell survival (Sivachandran et al., 2012).

In order to find the mechanism by which PML expression is lost in gastric cancer, we analyzed PML

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mutations in 50 formalin-fixed paraffin-embedded tissue blocks from gastric carcinoma tumors.

## Materials and Methods

### Sample collection

Fifty formalin-fixed paraffin-embedded tissue blocks with confirmed pathology of gastric adenocarcinoma were collected from Tehran hospitals.

### DNA extraction

Two 10  $\mu\text{m}$  thick sections were cut and placed into an Eppendorf tube using sterile toothpicks. After cutting of each block, the blade was cleaned with xylene to avoid contamination of paraffin wax block to another. Then 1 ml of xylene was added to each sample specimen and samples were placed on a rocker with gentle shaking for 90 minutes in 65°C to dissolve the paraffin. Afterwards, the sample was spun at 14,000 rpm or 16000 x g in a microcentrifuge for 3 minutes. Xylene supernatant was carefully withdrawn and disposed. Then 800  $\mu\text{l}$  of 100% ethanol (v/v) was added to each sample and samples were spun for 3 minutes at 14,000 rpm in microcentrifuge. Ethanol supernatant was removed and this step was repeated with 70% and 50% ethanol (v/v) solutions respectively. Tumor samples digestion with proteinase K (20 mg/ml; Promega) was carried out in 200  $\mu\text{l}$  digestion buffer (10 mM Tris-HCl, pH 8.0; 50 mM KCl; 1.5 mM MgCl<sub>2</sub>; 0.5% Tween 20) at 55°C for 48 hours on a rotating wheel apparatus, according to a method described previously with only slight modifications (Wang et al., 1996). Phenol-chloroform extraction and sodium acetate precipitation were also carried out to purify the DNA.

### Polymerase Chain Reaction (PCR)

PCR reactions were performed using 14 sets of primers designed to amplify the entire PML coding sequence and intron-exon boundaries as described previously (Gurrieri et al., 2004b). The total PCR volume was 25  $\mu\text{L}$  containing 1  $\mu\text{L}$  genomic DNA, PCR set and Taq enzyme (Fermentas, Burlington, ON, Canada). PCR conditions were as described previously (Gurrieri et al., 2004b). PCR products were then subjected to single strand conformation polymorphism (SSCP)/ silver staining assay as well as Conformation sensitive gel electrophoresis (CSGE).

### Restriction enzyme digestion

PCR products greater than 400 bp were digested with appropriate restriction enzymes such as MboII or EcoR I based on the exon sequence.

### Single strand conformation polymorphism (SSCP)/ silver staining assay

SSCP/ silver staining assay was performed according to the method described in previous studied with minor modifications (Wang et al., 1996). In brief, 5  $\mu\text{l}$  of PCR product was added to 5  $\mu\text{l}$  of the formamide loading buffer and then were denatured at 96°C for 12 minutes and the cooled denatured mixture was loaded onto non-denaturing 15% polyacrylamide (30:1 acrylamide:bisacrylamide)/ 10% glycerol gels cast. Samples were run in 1 x TBE at

175 volts for 15 hours. Gels were immersed in 10% ethanol for 5 minutes, 1% nitric acid for 5 minutes, stained in 0.1% silver nitrate solution for 10 minutes, and developed in sodium carbonate solution (60 mg/ml) and formaldehyde (1.2  $\mu\text{l}/\text{ml}$ ). Development was stopped by soaking the gel in 10% acetic acid for 5 minutes.

### Conformation sensitive gel electrophoresis (CSGE)

CSGE analysis was performed according to the method described in previous studies with minor modifications (Bahram et al., 2009).

### Sequencing

Abnormally shifted bands were directly sequenced using the ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

## Results

### General statistical information

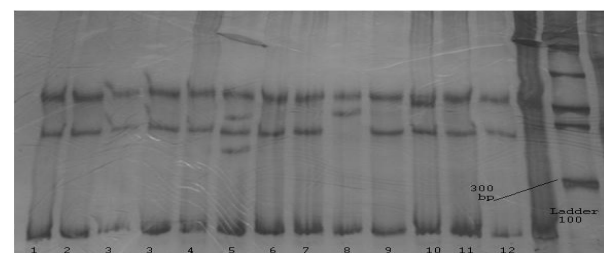
Fifty gastric adenocarcinoma samples including 6 diffuse type (4 mucinous) entered the study. Among them, 41 (82%) were male and 9 (18%) were female with an age range of 35-85 years (mean  $\pm$  SEM 58  $\pm$  14.6). Nearby lymph node was involved in 58% and penetration to the third layer of stomach was detected in 22% of patients. Paraclinic studies revealed omentum involvement in 1 patient and distant metastasis to colon, pancreas and liver in 3, 2 and 1 patient respectively.

### SSCP/ silver staining assay and CSGE analysis

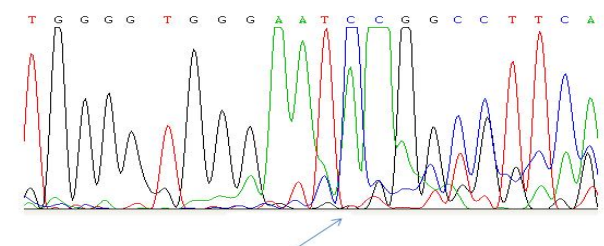
The PCR fragment corresponding to each exon was loaded on to non-denaturing polyacrylamide gels. The pattern of bands was compared to normal controls. Figure 1 shows that SSCP banding patterns were shifted in 2 samples corresponding to patients.

### Sequence analysis

Sequence analysis of 3 samples corresponding to exon 5 PCR products showed a novel sequence



**Figure 1. SSCP gel of Exon 5 of PML Gene.** Lanes 5 and 8 show abnormally shifted bands



**Figure 2. Sequence Analysis of abnormal bands Corresponding to exon 5 Segment**

variant of unknown significance localized in intron 5 (c.1398+84delA) (Figure 2).

## Discussion

Vast bulk of evidence supports the role for PML as a tumor suppressor. Its inactivation has led to survival and proliferative advantage to cells of diverse histologic types (Guo et al., 2000), while loss of its function disrupts cellular senescence in response to oncogenic stimuli (Pearson et al., 2000). In addition, PML modulates important tumor-suppressive pathways for instance the p53 and Rb pathways, which are disrupted in PML deficient cells (Gurrieri et al., 2004b). PML mutations have been found in APL patients by SSCP technique (Gurrieri et al., 2004b). Although the genetic mechanisms responsible for development of leukemia have been regarded as different from solid tumors, it has been revealed that many of the genes involved in the leukemia associated translocations are also expressed in non-hemopoietic cells and direct crucial survival and proliferation pathways (Gurrieri et al., 2004a).

SSCP analysis has been demonstrated as a simple and effective technique for the detection of single base substitutions with the optimal size fragment for sensitive base substitution detection being less than 200 bp (Sheffield et al., 1993). An optimized SSCP analysis has been suggested to be used to efficiently screen for mutations in a large gene (Ravnik-Glavac et al., 1994). With the use of SSCP and CSGE techniques we could not find any deleterious mutations of the PML gene in 50 gastric cancer samples. In another study of mutation screening of the entire coding region and intron-exon boundaries of the PML gene in large sample of diverse tumor types (but not including gastric cancer samples), few sequence variants were found in tumor samples (including melanoma, lung cancer, and lymphoma samples). Interestingly, none of these sequence variants affected PML protein expression. So it has been suggested that although loss of PML protein is a common event in human cancers of various histologic origins, it may be lost through proteasome-dependent mechanisms (Gurrieri et al., 2004a).

Previous reports have shown that although PML is more commonly completely lost in advanced cancers, even in early stages of tumorigenesis it has been lost (Gurrieri et al., 2004a). However, in our study we could not detect any deleterious mutation even in patients with advanced tumor stages. In addition, PML protein expression has been shown to be lost in certain tumor types and not in others (Gurrieri et al., 2004a). As gastric cancer is among cancers in which PML expression is frequently lost (Lee et al., 2007), our results suggest that PML loss of function in gastric cancer is due to a mechanism rather than gene mutation. As proposed previously, proteasome dependent degradation might be a possible mechanism.

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