OCT4B1, a novel spliced variant of OCT4, is highly expressed in gastric cancer and acts as an antiapoptotic factor

Malek H. Asadi1, Seyed J. Mowla1, Fardin Fathi2, Ahmad Aleyasin3, Jamshid Asadzadeh1 and Yaser Atlasi1

1 Department of Molecular Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran
2 Center for Cellular and Molecular Research, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran
3 Department of Medical Genetics, National Institute for Genetic Engineering and Biotechnology, Tehran, Iran

The octamer-binding transcription factor 4 (OCT4) is involved in regulating pluripotency and self-renewal maintenance of embryonic stem cells. Recently, misexpression of OCT4 has been also reported in some adult stem as well as cancer cells; a finding which is still controversial. In addition to the previously described spliced variants of the gene (e.g., OCT4A and OCT4B), we have recently identified a novel variant of the gene, designated as OCT4-B1. In this study, we investigated a potential expression and function of OCT4B1 in a series of gastric cancer tissues and a gastric adenocarcinoma cell line, AGS. Using the Taqman real-time PCR approach, we have detected the expression of OCT4B1 in tumors with no or much lower expression in marginal samples of the same patients (p < 0.002). We have also analyzed the effects of OCT4B1 knock-down in AGS cell line treated with specific siRNA directed toward OCT4B1. Our data revealed that interfering with the expression of OCT4B1 caused profound changes in the morphology and cell cycle distribution of the cells. Furthermore, down-regulation of OCT4B1 significantly elevated the relative activity of caspase-3/caspase-7 and the rate of apoptosis in the cells (more than 30%). All together, our findings suggest that OCT4B1 has a potential role in tumorigenesis of gastric cancer and candidates the variant as a new tumor marker with potential value in diagnosis and treatment of gastric cancer.

On the basis of the cancer stem cell (CSC) hypothesis, a subpopulation of cells within tumors is responsible for the sustained growth and propagation of tumors. CSCs are also believed to be the main reason for the tumor relapse and resistance to therapy. These cells share various characteristics similar to normal tissue-specific adult stem cells, including self-renewal potential. Based on this hypothesis, CSCs are originating either from the dysregulated self-renewal control in normal adult stem cells or through reprogramming of the somatic progenitor or differentiated cells within the tissue.1–5

The octamer-binding transcription factor 4 (OCT4, also known as OCT3 and POU5F1) belongs to a family of transcription factors containing the POU DNA-binding domain. The encoded protein acts as a master self-renewal regulator in embryonic stem cells and plays a critical role in maintaining the pluripotent state of stem cells. OCT4 expression is strongly repressed following stem cell differentiation.6–10 Several recent studies have demonstrated the unexpected expression of the OCT4 gene in some human cancer cell lines and tissues,11–18 including gastric cancer.19

The human OCT4 gene can potentially encode three spliced variants, designated as OCT4A, OCT4B and the newly discovered OCT4B1.20 OCT4A is primarily localized within the nucleus of the embryonic stem cells, where it sustains the self-renewal and pluripotency properties of the cells. In contrast, OCT4B is mainly located within the cytoplasm of somatic cancer cell lines and apparently lacks any self-renewal regulatory role.21,22 In addition to its expression in somatic cells, OCT4B1 is highly expressed in ES/EC cells and is rapidly down-regulated upon induction of differentiation.20 Based on our previous data on SSEA3(+) stem cells, there is a potential correlation between the expression of OCT4B1 and the pluripotent/undifferentiated state of ES/EC/CS cells.20

While the expression of OCT4 has been detected in several cancer cell lines and tumors, little information exists about the discriminative expression and function of OCT4 spic variants in stem/cancer cells. Indeed for the newly discovered variant of OCT4, OCT4B1, almost nothing is known about its expression and function in cancer tissues and cell lines. Thus, we investigated the potential expression of OCT4B1 in a series of gastric cancer samples. We further investigated the possibility of its biological role by knocking down OCT4B1 expression within a gastric adenocarcinoma cell line, AGS, using RNAi technology.
Material and Methods

Clinical samples collection

Tumor and nontumor (apparently normal tissues from the margin of same tumors, as control) surgical specimens from 36 patients with gastric adenocarcinoma were provided by the Iran National Tumor Bank. The obtained samples had been immediately snap-frozen in liquid nitrogen and had been stored in −185°C until being used for RNA extraction. A record of clinicopathological parameters, including grading and staging, for each patient were received along with each sample (Supporting Information Table 1). The experiment design was approved by the Ethics Committee of Tarbiat Modares University.

Cell lines and cell culture

The gastric adenocarcinoma cell line AGS was obtained from the national cell bank of Iran (Pasteur institute of Iran, Tehran) and was cultured in RPMI-1640 (Gibco), supplemented with penicillin/streptomycin (100 U/ml and 100 µg/ml, respectively) and 10% fetal bovine serum, at 37°C in a humidified atmosphere of 5% CO₂. The human EC cells, NTERA2 (NT2), were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with high concentration of glucose (4500 mg/l), supplemented with 10% fetal bovine serum at 37°C (humidified) and 5% CO₂.

RNA extraction and real-time PCR

Total RNA was isolated from the homogenized tissue specimens, using RNX plus™ solution (Cinnagen, Tehran, Iran) according to the manufacturer’s instructions. Purity and integrity of the extracted RNA was measured by UV spectrophotometry (260/280-nm ratio) as well as visual observation of samples on agarose gel electrophoresis. RNase-free DNase (Fermentase, Lithuania) treatment of total RNA was performed according to the manufacturer’s instructions. The first strand of cDNA was synthesized at 42°C for 60 min in the presence of 1 µg RNA, 200 U/µl MMLV reverse transcriptase (Fermentase, Lithuania), 20U RNase inhibitor, dNTP mix (final concentration of 1 mM) with oligo(dT) 18 priming in a 20 µl reaction.

Specific primers and probes were designed for OCT4A, OCT4B, OCT4B1 and GAPDH (GenBank accession numbers: NM_002701, NM_203289, EU518650 and NM-002046, respectively) using AlleleID 4.0 and Gene Runner software (Table 1).

Real-time PCR reactions were performed using Taqman method with premix Ex Taq™ (Takara) on an ABI 7500 real-time quantitative PCR system. PCR reaction mixture (12.5 µl premix Ex Taq™, 0.2 µM forward primer, 0.2µM reverse primer, 0.1µM TaqMan probe, 0.5 µl ROX reference dye II, 2 µl template and 8 µl H₂O) was prepared and PCR was performed with the following cycling conditions: initiation at 94°C for 30 seconds, amplification for 45 cycles with denaturation at 94°C for 5 seconds, annealing and extending at 60°C for 34 seconds. To determine the reaction efficiencies for each primer pair and the corresponding probe, standard curves were plotted by using serial dilutions of NT2 cDNA. All reaction efficiencies were measured to be close to 100%.

All experiments were conducted in duplicate or triplicate. Group-wise comparison and statistical analysis of relative expression results of real-time PCR were carried out by REST 2008 (Relative Expression Software Tool, V2.0.7, Corbette Research). SPSS 17.0 for windows (Chicago, IL) was also used to plot the charts and to analyze the correlation between the expression of each variant and the tumor state and grade of the samples.

Immunocytochemistry

AGS and NT2 cells were fixed in 4% paraformaldehyde for 15 min at room temperature. Cells were then permeabilized in 0.25% Triton X-100 [0.1% Tween-20 in phosphate buffered saline (PBS)] for 10 min and blocked in 1% BSA in PBST for 30 min. The cells were then incubated in the diluted first antibody (an affinity-purified goat polyclonal anti-OCT4 antibody, sc-8629; Santa Cruz Biotec) in PBST containing 1% BSA for 1 hr at room temperature. The antibody reacts with the C-terminal of OCT4 protein and hence recognizes both OCT4A and OCT4B variants. After washing the cells three times with PBS, cells were incubated with the secondary antibody (anti-goat IgG; Sigma-Aldrich) at dilutions 1:100 in 1% BSA for 1 hr at room temperature. The cells were then washed 3 times with PBS, counterstained with Geimsa and visualized under a fluorescent microscope (Nikon, Germany). For negative control samples, all conditions were kept the same, except that the primary antibody was omitted.

Table 1. Designed primers and probe for OCT4 variants and GAPDH

<table>
<thead>
<tr>
<th>Gene</th>
<th>Designed primer</th>
<th>Sequence</th>
<th>Amplican length</th>
</tr>
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<tbody>
<tr>
<td>OCT4 A</td>
<td>F</td>
<td>CGCAAGCCCTTCATTCAC</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CATCACCTCCACACCTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>CTTCGATTTGCCCTTCTGCC</td>
<td></td>
</tr>
<tr>
<td>OCT4 B</td>
<td>F</td>
<td>CAGGGAATGGGTTGAATGC</td>
<td>177</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>AGCGAGAACAGCTGTAAGAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>AGTATGTTGGCAGTGGAGGCA</td>
<td></td>
</tr>
<tr>
<td>OCT4 B1</td>
<td>F</td>
<td>GGTTCTATTTGTTGGTTC</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TCCCTCTCCACTCTCCCTCA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>ATTTGACCGCCTCCTCCCTCAAG</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>F</td>
<td>GTGACACTAGAAGATAGACAA</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CATGAGTCCTCCACGATACC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>CCTCAGATCATCAGAATGCTCCTCG</td>
<td></td>
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</table>

Abbreviations: F, forward primer; R, reverse primer; P, probe.
RNA interference
Two specific siRNAs for OCT4B1 suppression in AGS cell line and an irrelevant siRNA (IR-siRNA) with no complementary target sequence more than 16 out of its 21 mer length within human genome were designed by the siRNA Selection Program (Whitehead Institute for Biomedical Research; http://jura.wi.mit.edu/) and synthesized by MWG company (Germany).

The sequences of the siRNAs are as followed:

Oct4B1-siRNA1 Target sequence: AAG GAG TAT CCC
TGA ACC TAG
Sense: (GGA GUA UCC CUG AAC CUA G)dT dT
Antisense: (CUA GGU UCA GGG AUA CUC C)dT dT
Oct4B1-siRNA2 Target sequence: AAG AGG TGG TAA
GCT TGG ATC
Sense: (CAG GUG GUA AGC UUG GAU C)dT dT
Antisense: (GAU CCA AGC UUA CCA CCU C)dT dT

The above siRNAs were designed based on the Exon2b sequence of OCT4B1, which discriminates it from the other variants of OCT4.

IR:
Sense: GCGGAGAGGCUUAGGUGUAdTdT
Antisense: UACACCUAAGCCUCUCCGdTdT

One day before siRNAs transfection, 2 × 10^4 cells per well (30–50% of confluency at the time of transfection) were cultured on six-well plates in growth medium without antibiotic. siRNAs were introduced into the cells, using the Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen).23

Briefly, 5μl of siRNA (25μM) solution and 4.5μl RNAiMAX reagent were diluted in 250μl Opti-MEM (Invitrogen) and incubated for 10 min in room temperature. The mixture was then added to the cells in a final volume of 2.5 ml. Cells were further incubated for 3 days at 37°C in a 5% CO₂ incubator.

Cell cycle analysis
For cell cycle analysis, cells were washed with PBS and trypsinized with 0.025% trypsin-EDTA to yield single cell suspensions. Then cells were stained with 50 μg/ml propidium iodide solution containing 0.1% Triton X-100 and sodium citrate as described elsewhere.24

The single cell suspensions were then used for flow cytometric (Partec, Germany) analysis. Cell cycle profiles were analyzed using Partec Flomax software.

Apoptosis analysis by annexin-V-FLUOS staining kit
Apoptosis was measured by detecting the exposed phosphatidylserine in the cell membrane, using the Annexin-V-FLUOS and propidium iodide kit (Roch). The cells were then analyzed on a flow cytometer and typical histograms of apoptotic versus nonapoptotic and necrotic cells were generated.

Caspase assay
Caspase-3 and caspase-7 activities were measured using Caspase-Glo 3/7 reagent (Promega). Caspase-Glo 3/7 reagent added to the cell suspension aliquots in quadruplicate, and incubated at room temperature for 1 h. The luminescence of each sample was then measured by a Luminometer (Berthold, Germany).

Results
OCT4B1 is highly expressed in gastric adenocarcinoma
To study the possibility of OCT4B1 expression in gastric adenocarcinoma, and to compare its expression level with those of the OCT4A and OCT4B variants, we carefully designed forward and reverse primers and probes for specific amplification of each variant. The primers also designed in a way to avoid nonspecific amplification of OCT4 pseudogenes, which have highly similar sequences to OCT4A. Furthermore, the identity of the amplified products was confirmed by direct DNA sequencing (data not shown). The relative expression of OCT4 spliced variants was compared between tumor and nontumor marginal tissues obtained from the same patients. Expression of GAPDH was used in all samples as an internal control, for normalization of probable sampling errors.

Our data revealed that OCT4B1 is expressed in both tumor and nontumor samples of stomach. OCT4B1 expression was detected in 86.1% (31/36) of the tumor samples and 55.5% (20/36) of the marginal counterparts of the same tumors (Fig. 1a). However, the level of expression was much higher in the tumor samples compared to the apparent normal tissues obtained from the margin of same tumors (p < 0.002; Fig. 1b). Moreover, in the tumor group, OCT4B1 expression was much higher in the high-grade tumors compared to the low-grade ones (p < 0.05; Fig. 1c).

Similar observations were made for OCT4A and OCT4B variants. OCT4A expression was detected in 91.67% (33/36) of tumor samples and 58.33% (21/36) of marginal samples. Moreover, its expression level was several folds higher in the tumor group compared to that of nontumor (marginal) group (p < 0.001; Fig. 1d and 1e). The level of expression was also significantly higher in high-grade tumors compared to the low-grade ones (p < 0.01, Fig. 1f).

Similarly, the expression of OCT4B was detected in 91.67% (33/36) of tumor samples and 44.5% (16/36) of non-tumor ones. OCT4B showed a significant overexpression in the tumor group in comparison to the marginal samples (p < 0.001; Figs. 1g and 1h). In contrast to other two variants, while the expression of OCT4B was apparently higher in the high-grade tumors, the difference was not statistically significant (Fig. 1k).

All three variants of OCT4 are expressed in the AGS gastric adenocarcinoma cell line
We used AGS, a gastric adenocarcinoma cell line, as a model system to evaluate the effects of OCT4B1 knock-down on the
cells’ behavior. Accompanying each set of experiments, the embryonic carcinoma cell line, NT2, was also employed as a positive control. As shown in Figure 2a, all three variants of the gene are expressed at the mRNA level in both cell lines. Since there is not yet a specific antibody against OCT4B1, and it is not even clear whether the variant is translated at the protein level, we examined the expression of the other two variants of OCT4 at protein level. Using a polyclonal antibody capable of detecting both OCT4A and OCT4B isoforms, we determined the expression and subcellular localization of these variants by immunocytochemistry. While a nuclear signal was easily detectable in NT2 cells (Fig. 2e), the immunoreactivity signal was primarily cytoplasmic in AGS cells (Fig. 2c). The latter finding suggests that despite expression of OCT4A at mRNA level, its translated form is either not produced or stably maintained in AGS cell line.

**Morphological changes of AGS cells after OCT4B1-siRNA transfection**

The level of OCT4B1 expression in AGS cells treated with the specific siRNA against the variant was dramatically reduced in a magnitude of 10 times, compared to the cells transfected with a scrambled siRNA control (Fig. 2d). This reduction in expression correlated with changes in the morphology of the cells, where the transfection of OCT4B1 siRNA resulted in a more epithelial-like appearance, as opposed to the more mesenchymal phenotype observed in the control cells.
with an irrelevant (IR)-siRNA (Fig. 3a). Three days following the suppression of OCT4B1, the morphology of the cells was dramatically altered; while there were no obvious morphological changes in the cells treated with the IR-siRNA, demonstrated a primarily cytoplasmic signal for translated form of OCT4 in AGS cells (c). In contrast, NT2 cells, an embryonic carcinoma cell line, showed a nuclear immunoreactivity signal for OCT4 (e), (b) The phase contrast invert microscopy counterpart image of the cells in C, (d) DAPI staining of the cells in (c), illustrating the nuclei of the cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

**Cell cycle alterations following OCT4B1 knock-down in AGS cells**

Cell cycle distribution of the AGS cells transfected with OCT4B1-siRNA and IR-siRNA was determined by flow cytometry, 3 days after transfection. Compared to the cells treated with IR-siRNA, there existed only two peaks of counted cells in OCT4B1-siRNA transfected group (Fig. 4).
Moreover, the fraction of the cells appearing in sub-G1, a characteristic feature of apoptotic cells, constituted about 50% of the total cells. The remaining cells had DNA contents higher than that of normal cells, suggesting that the cells were multinuclear, probably containing two or more nuclei.

**Elevated rate of apoptosis in AGS cells treated with OCT4B1-siRNA**

Flow cytometry analyzes of the cells stained with Annexin-V-FLOUS and propidium iodide demonstrated that by 3 days after OCT4B1-siRNA transfection up to 39% of the cells had been undergone apoptosis, in comparison to the 13% of the cells transfected with IR-siRNA. Furthermore, the ratio of apparently necrotic cells increased to 20% of the total cells in OCT4B1-siRNA treated cells, a five-times increase compared to that of IR-siRNA transfected cells (Fig. 5a and 5b).

Similar observations were made with Caspase activity assay experiment in which relative activities of caspase-3 and caspase-7 were measured using a luminescence-based assay. OCT4B1-siRNA treated cells showed a significant elevation in the activities of caspase-3 and caspase-7, in contrast to the cells treated with IR-siRNA (p < 0.01, Fig. 5c).

**Discussion**

The transcription factor OCT4 is a well-known embryonic stem cell marker, with a key role in regulating stemness state, self-renewal and pluripotency of stem cells. Recent reports showing the expression of OCT4 in cancer cell lines and tissues, appeared to be highly controversial. Several other reports claimed that OCT4 is not expressed in cancer cell lines and somatic stem cells and its expression in the earlier reports is prone to artifacts, probably generated by OCT4 pseudogene transcripts. One other possibility for the contrary results presented by these reports is their failure to discriminate among the expressions of different variants of OCT4.

In an earlier work to address the aforementioned issue, we designed specific PCR primers and probes to distinguish the expression of different variants of OCT4 (OCT4A and OCT4B) in a wide spectrum of tumor vs. stem cell lines. Our data revealed that OCT4A is primarily expressed in ES cell lines, where the protein is primarily located in the nuclei of the cells. In contrast, the OCT4B variant is preferentially expressed in tumor cell lines, and the encoded protein is mostly located within the cytoplasm of the cells. This distinctive expression pattern and subcellular distribution of OCT4 variants accounts for some earlier inconsistency and highlighted the necessity of defining the variant of OCT4 when addressing the expression of the gene in different cell lines and tissues.

Furthermore, we discovered a novel variant of OCT4, designated as OCT4B1, which is highly expressed in stem cells and some tumor cell lines, and down-regulated upon the induction of differentiation. The differential pattern of OCT4B1 expression in differentiated and nondifferentiated stem cell lines suggests a potential biological role for the variant in stem as well as tumor cell lines.

To investigate the possibility of OCT4B1 involvement in tumorigenesis, we investigated here the expression of the variant in tumor vs. nontumor marginal tissues of 36 patients with gastric adenocarcinoma. Our results revealed that OCT4B1 is expressed in most samples of gastric adenocarcinoma, where its level of expression is highly elevated in tumor tissues compared to that of nontumor samples. Interestingly, the level of OCT4B1 expression was significantly higher in high-grade tumors in contrast to that of low-grade ones. This finding suggest that (1) OCT4B1 can be regarded as a new tumor marker for gastric, and probably other tumors and (2) in contrast to the OCT4B variant, OCT4B1 had a better correlation with the state of differentiation of the cells and could better predicts the degree of malignancy of tumors. This finding is in accordance with our previous report that the expression of OCT4B1 is sharply down-regulated upon the induction of differentiation of stem cell
lines. The low expression of OCT4 variants in apparently nontumor marginal tissues of the same patients could be due to the penetration of cancer cells into their apparently normal margins. An alternative interpretation is that the detected OCT4 expression is due to the presence of the normal adult stem cells, which do exist in all normal tissues. Indeed, the expression of OCT4 at mRNA and protein levels has been already reported in several tissue-specific human adult stem cells.

To investigate whether the upregulation of OCT4B1 in tumor samples has a causative tumorigenic role, we knocked-down the expression of the variant by using two separate sets of specific siRNAs in a gastric adenocarcinoma cell line, AGS. Successful inhibition of OCT4B1 in AGS cells, as confirmed by real-time RT-PCR, gave rise to the formation of some multinuclear giant-like cells and a significant increase in the rate of apoptosis. The latter findings suggest that OCT4B1 has a causative role in tumorigenesis and that it could be regarded as a potential therapeutic target to combat cancer.

One hallmark of the OCT4B1 knockdown in AGS cells was a transformation of the treated cells into a multinuclear giant-like morphology. The morphological changes in the AGS cells might be a mechanism to protect some cells from apoptosis or senescence, a hypothesis that need to be experimentally validated. However, based on previous reports, following the induction of apoptosis, cells mutated for p53 could enter into an abnormal cycle that leads toward the formation of giant polyplody cells. On contrary, cells with wild type p53 exhibited mitotic arrest as well as programmed cell death. Despite having apparently wild type p53, AGS cells change their morphology into giant cells following treatment with anticancer drug, taxol. Therefore, this property of AGS cells suggests that inhibition of OCT4B1 may induce cell death in most cells, but giant-cell transformation in a portion of cells could help them to escape apoptosis.

It is not yet clear whether OCT4B1 exerts its functions at the mRNA or protein level. Insertion of intron 2 generates a frame-shift in the sequence of OCT4B1 and the formation of a putative 115 amino acids truncated form of the protein. Due to the lack of a specific antibody against OCT4B1, the possible generation of a stable protein form of the variant is still unclear. Comparison of the amino acid sequences of OCT4B1 and OCT4B showed the identity of amino acids 1–80 between the two proteins. They have similar N-terminal domain and a part of the POUs domain, comprising amino acids 43 through 80. However, OCT4B1 lacks the rest of the POUs domain as well as the POUH and C-terminal transactivation domains. Determining the expression of OCT4B1 at the protein level along with its subcellular distribution within stem and cancer cells would shed more light on clarifying the biological role of OCT4B1 in stem and cancer cells. In this regard, our primary data suggests that OCT4B1-HA is translated and localized in the cytoplasm of the transfected cells (own unpublished observations).

In conclusion, for the first time our data revealed the up-regulation of OCT4B1 in gastric adenocarcinoma and that the variant is contributing in tumorigenesis process as an antiapoptotic factor. Hence, OCT4B1 can be considered as a novel tumor marker with potential diagnostic, prognostic and therapeutic value.

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References


