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Oct4, a Novel Marker for Human Gastric Cancer

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Background and Objective: Octamer-4 (Oct4), a transcription factor involved in regulating human embryonic stem cells (ESCs), may play a role in tumorigenesis. Since little is known about the efficacy of Oct4 as a potential biomarker for gastric cancer (GC), we investigated its expression in GC tissues and its relationship to various clinicopathological parameters.

Methods: Primary tumor tissues and matching, adjacent non-cancerous tissues were obtained from 62 GC patients, and Oct4 expression was examined by reverse transcription-PCR (RT-PCR) and real-time PCR. Twenty biopsy specimens of atrophic gastritis and gastric ulcer individually were collected as control. To detect Oct4 expression in the paired GC and non-cancerous tissues at the protein level, Western blotting and immunohistochemistry (IHC) were employed. Correlation analyses were conducted to assess the relationship between Oct4 expression and clinicopathological parameters.

Results: Oct4 expression levels were higher in GC tissues compared to matching, adjacent non-cancerous tissues, atrophic gastritis and gastric ulcer tissues. Additionally, Oct4 expression in GC tumors correlated with their differentiation status, but not with patient age or gender, tumor size, TNM stage, depth of invasion, or the presence of lymph node metastasis.

Conclusions: Oct4 may be a potential biomarker for the initiation, progression, and differentiation of human GC.
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KEY WORDS: **Oct4; stem cells; gastric cancer**

INTRODUCTION

Gastric cancer (GC) is the fourth most common malignancy and the second leading cause of cancer-related mortality worldwide. The origin of cancer has been attributed to stem cell maturation and de-differentiation, a cellular process where terminally differentiated cells revert to earlier developmental stages [1–5]. Octamer-4 (Oct4), a transcription factor expressed in both embryonic and adult stem cells, has been associated with the pluripotency, proliferative potential, and self-renewal properties observed in embryonic stem cells (ESCs) and germ cells [6]. Many cancers, including breast, brain, and bladder cancers, have been shown to contain tumor-initiating stem cells, also known as cancer stem cells (CSCs) [7–10]. These Oct4-expressing CSCs possess self-renewal capabilities and are derived from dys-regulated self-renewal pathways in normal stem cells or early progenitor cells [1–5,11].

Specific knockdown of Oct4 in ESCs and embryonic cancer cells results in the onset of differentiation and loss of pluripotency [12,13]. Other studies suggest that over-expression of Oct4 in epithelial tissues may lead to dysplasia by inhibiting progenitor-cell differentiation, an effect similar to that of Oct4 in ESCs [14,15]. Additionally, Oct4 increases the malignant potential of ESCs in a dose-dependent manner [16]. All of these studies suggest that Oct4 may play a role in oncogenesis.

Although Oct4 expression has been extensively studied in germ cell tumors [17], little is known about its expression in solid tumors. The present study was designed to investigate the expression of Oct4 in human GC and to determine the correlation between Oct4 expression and various clinicopathological parameters.

MATERIALS AND METHODS

Subjects

Informed consent was obtained from 62 GC patients who underwent surgery at the First People's Hospital of Zhenjiang or the Affiliated Hospital of Jiangsu University in Zhenjiang, China. All patients voluntarily agreed to participate in the study under the terms proposed by the local Ethical Committee. Surgical specimens were collected between January 2007 and January 2009 from 38 males and 24 females, ranging from 35 to 86 years of age (median, 58 years).

Abbreviations: GC, gastric cancer; RT-PCR, reverse transcription-PCR; CSCs, cancer stem cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ESCs, embryonic stem cells; FFPE, formalin-fixed paraffin-embedded; TBS, Tris-buffered saline; IHC, immunohistochemistry.

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Twenty biopsy specimens of atrophic gastritis and gastric ulcer individually were collected by gastrointestinal endoscopic examination. All the samples were confirmed by pathologic examination. Patients participating in the study had not received preoperative treatment. Resected tumor tissue samples and matched adjacent noncancerous tissue samples (located more than 5–10 cm away from the primary site) were sectioned and fixed in 10% formalin for immunohistochemistry (IHC). Remaining paired sections were snap-frozen in liquid nitrogen, and stored at −80°C until analysis. Histopathological grade was defined according to the International Union Against Cancer TNM Classification (5th Edition) and the World Health Organization classification [18]. Differentiation status was assessed according to standard pathological criteria.

RNA Extraction

Tissue sections were homogenized with Trizol reagent, and 200 μl chloroform was added to the Trizol homogenate. The preparations were then centrifuged at 12,000*g* for 15 min at 4°C, and the upper aqueous layer was transferred to a clean Eppendorf tube containing an equal volume of isopropanol. The mixtures were added to RNAfast200 mini-columns (Fastagen Biotech, Shanghai, China) and processed according to the protocol provided by the manufacturer. Finally, total RNA was dissolved in RNase-free water and the quality of RNA was evaluated by gel electrophoresis. RNA concentrations were measured by optical density (260 nm) and the preparations stored at −80°C until further analysis.

PCR Primers

To eliminate potential non-specific amplification of contaminated genomic DNA, primers for human Oct4 and β-actin genes were designed to be intron-spanning, as shown in Table I.

Reverse Transcription-PCR

cDNA was reverse transcribed using a kit provided by Toyobo Biologics (Osaka, Japan) and stored at −20°C until further analysis. Oct4 primers 1 and 2 and β-actin primers 5 and 6 were used to amplify the cDNA fragments. All reverse transcription PCR reactions were carried out on an ABI 2720 Thermal Cycler (Applied Biosystems, Foster City, CA) using the following cycling conditions: 94°C for 30 sec; 60°C (Oct4) or 56°C (β-actin) for 30 sec; 72°C for 30 sec; and a final extension at 72°C for 10 min. PCR amplification was performed for either 35 (Oct4) or 30 (β-actin) cycles to yield the 397-base pair (Oct4) and 265-base pair (β-actin) products. PCR products were separated on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light.

Real-Time PCR

All PCR reagents were purchased from TaKaRa Bio, Inc. (Shiga, Japan). Oct4 primers 3 and 4 and β-actin primers 5 and 6 were used to amplify the cDNA fragments. To compensate for variations in the amount of input RNA and the efficiency of reverse transcriptase, β-actin mRNA was quantified, and results were normalized to these values.

All real-time PCR reactions were carried out on the Rotor-Gene 6000 Real-Time Fluorescence Thermal Cycler (Corbett Ltd, Sydney, Australia) using the following cycling conditions: initiation at 94°C for 10 min; amplification for 35 cycles with denaturation at 94°C for 30 sec, annealing at 60°C (Oct4) and 56°C (β-actin) for 30 sec, and elongation at 72°C for 30 sec. A final extension at 72°C was performed for 10 min, and then the samples were subjected to a final melting from 72 to 99°C. To obtain a standard curve, the PCR products were subcloned into the pGEM-T vector (Promega, Madison, WI) according to the manufacturer's instructions. After a series of dilutions, standard data were analyzed by real-time analytical software (Rotor-Gene, Corbett Robotics, Inc, San Francisco, CA). Both the standards and samples were processed in duplicate. To confirm the identity of the amplicons, melting curve analysis and agarose gel electrophoresis and ethidium bromide staining of the PCR products were accomplished.

Relative expression levels were determined by dividing the normalized value of each tumor sample by the corresponding normalized value of non-cancerous tissue and the other two control groups.

Western Blotting Analysis

For Western immunoblot analysis, frozen tissue samples were homogenized and lysed in modified RIPA buffer. The protein concentration was measured by a bicinchoninic acid protein quantification kit (Shen Neng Bocai, Shanghai, China) and samples were loaded on a 10% SDS–PAGE gel (25 μg). Following electrophoresis, the proteins were transferred to a BioTrace™ PVDF membrane (Pall Life Sciences, Ann Arbor, MI) and blocked in 5% skim milk for 1 hr at room temperature. The blots were probed with antibodies against Oct4 (SC-8629, Santa Cruz Biotechnology, Santa Cruz, CA) and GAPDH (KC-5G4; Kang Chen, Shanghai, China), and detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK), and visualized using the Typhoon 9400 Scanner (GE Healthcare, Piscataway, NJ).

Immunohistochemical Analysis

Formalin-fixed paraffin-embedded (FFPE) tissue sections (4-μm) were deparaffinized in xylene, rehydrated through a series of ethanol dilutions, and boiled for 10 min in citrate buffer (10 mM, pH 6.0). Endogenous peroxidase activity was suppressed by exposure to 3% hydrogen peroxide for 10 min. Slides were then blocked with 5% BSA (Boster Corp., Wuhan, China) and incubated with anti-Oct4 polyclonal antibody (SC-8629 Santa Cruz Biotechnology) for 1 hr at 37°C. A rabbit anti-goat secondary antibody was used according to the protocol supplied with the Boster ABC Staining System kit. Sections not probed with primary antibody were considered negative controls.

Statistical Analysis

All statistical analyses were carried out using SPSS 13.0 for Windows (Chicago, IL). Experiments were conducted in duplicate or triplicate. Positivity rates and differences in expression between these

TABLE I. Primers for Oct4 and β-Actin Genes

Gene	Primer sequence	Annealing temperature	Product (bp)
Oct4	Primer 1: For: 5′-TATACACAGGCCGATGTGG-3′ Primer 2: Rev: 5′-GTGCATAGTCGCTGCTTGA-3′	60	397
	Primer 3: For: 5′-TTGAGGCTCTGCAGCTTAG-3′ Primer 4: Rev: 5′-GCCGGTTACAGAACCACAC-3′	60	285
β-actin	Primer 5: For: 5′-CACGAAACTACCTTCAACTCC-3′ Primer 6: Rev: 5′-CATACTCCTGCTTGCTGATC-3′	56	265

groups were estimated using the chi-square test and the non-parametric Wilcoxon rank sum test, respectively. Correlations between Oct4 expression and clinicopathological parameters were also statistically analyzed. Probability values less than 0.05 were considered significant.

RESULTS

Over-Expression of Oct4 in GC Tissues

Given the existence of spliced variants of Oct4, we designed specific primers to amplify a fragment of Oct4. The oligonucleotide sequences of PCR primers and reaction parameters used in this study are listed in Table I. A 397 bp PCR product was amplified using primers 1 and 2 and the amplicon was confirmed to be Oct4 by DNA sequencing. Of 62 paired patient samples, 67.7% (42/62) of tumor tissue samples and 6.5% (4/62) of matched normal tissue samples expressed the Oct4 gene (Fig. 1A). Of the 20 cases of atrophic gastritis, 3 were positive for Oct4 expression (15.0%) (Fig. 1B) and 10.0% (2/20) was also positive in the samples of gastric ulcer (Fig. 1C). In the rare cases where Oct4 was detected in both the tumor and neighboring non-cancerous tissues, the median expression levels were 0.026 and 0.0084, respectively ($P = 0.0153$) (Fig. 1D). The comparative expression level of Oct4 in GC tissues was significantly higher than that in the atrophic gastritis and gastric ulcer groups ($P = 0.0341$, $P = 0.0246$) (Fig. 1D).

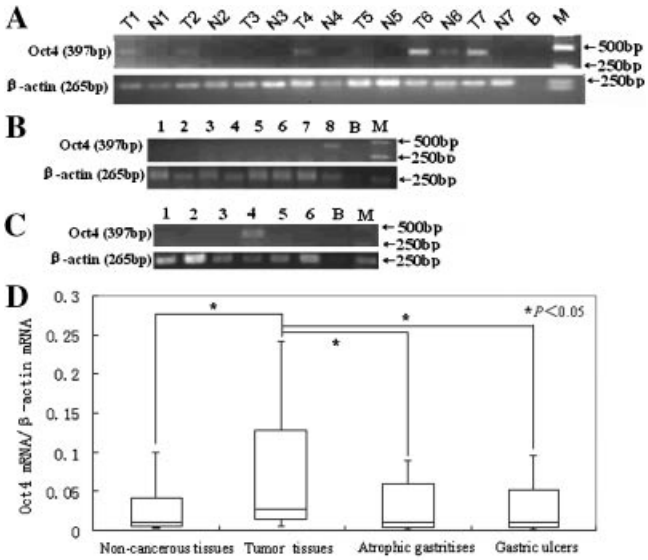

Fig. 1. Analysis of Oct4 expression in paired gastric tissues, atrophic gastritises and gastric ulcers. **A**: RT-PCR analysis of Oct4 mRNA expression in primary gastric tumors (T) and matching, adjacent non-cancerous tissues (N). **B**: Oct4 mRNA expression in atrophic gastritises. **C**: Oct4 mRNA expression in gastric ulcers. β-actin was used as an internal control. B, Water blank; M, DNA Marker (DL-2000). **D**: Comparison of relative expression levels of Oct4 mRNA among these groups, as determined by real-time PCR. Boxes indicate the percentiles. The lines in the boxes indicate the median values for the entities (0.026 for tumor tissues, 0.0084 for adjacent non-cancerous tissues, 0.0116 for atrophic gastritises, and 0.0107 for gastric ulcers). The differences in the relative expression levels among gastric cancer tissues, the adjacent non-cancerous tissues, the atrophic gastritises and gastric ulcers are significant ($P = 0.0153$, $P = 0.0341$, $P = 0.0246$, respectively).

Elevated Oct4 Protein in GC Tissues

Oct4 protein expression was assessed by SDS–PAGE followed by Western blot analysis using a polyclonal anti-Oct4 antibody. Seminoma tissue was used as a positive control. A 50-kDa band was detected in both the seminoma and tumor tissues. Weaker bands were detected in non-cancerous tissues; and in some cases, no Oct4 was detected (Fig. 2).

Distribution and Localization of Oct4

IHC using a polyclonal anti-Oct4 antibody was carried out to determine the tissue distribution and subcellular localization of Oct4 in paired gastric tumor and non-cancerous tissue sections. FFPE seminoma tissue sections were used as positive controls, as seminomas exhibit strong nuclear and faint cytoplasmic staining of the Oct4 protein (Fig. 3A) [19]. Oct4 was primarily localized in the nuclei and cytoplasm of the GC cells (Fig. 3B), with no immunoreactivity in the matching, adjacent non-cancerous cells (Fig. 3C). Moreover, the intensity of immunoreactivity was variable among Oct4-positive cells, suggesting that the tumor cells are heterogeneous in terms of their Oct4 expression. No immunoreactivity was observed in negative controls, which were incubated in the absence of primary antibody.

Correlation Between the Expression of Oct4 and Clinicopathological Parameters

Oct4 expression significantly correlated with the differentiation of the tumors. Poorly differentiated and undifferentiated GC tissues expressed higher levels of the transcription factor compared to moderately and well-differentiated GC tissues ($P = 0.0292$). Oct4 expression was not associated with any other clinicopathological factors, including patient age or gender, tumor size, TNM stage, depth of invasion, or the presence of lymph node metastasis. The relationship between Oct4 expression in GC and various clinicopathological parameters is summarized in Table II.

DISCUSSION

In the present study, we investigated the status of Oct4 in the paired GC tissues, matched adjacent non-cancerous tissues, biopsy specimens of atrophic gastritis and gastric ulcer and explored its relationship to clinicopathological parameters. Oct4, a transcription factor in the POU family of proteins, has been implicated in stem-cell pluripotential, cell type-specific terminal differentiation, and early embryonic development [20–24]. Okamoto et al. [22] demonstrated a positive correlation between high level of Oct4 protein and an increase in the malignant potential of murine ESC-derived tumors. Oct4 plays a significant role

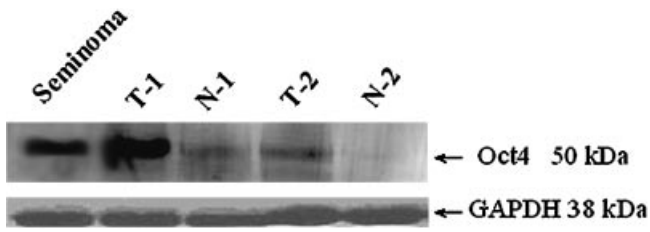

Fig. 2. Oct4 protein expression in the paired gastric tissues as measured by Western blotting. Total proteins were extracted from seminoma tissue (used as positive control), tumor tissues (T1 and T2), and the matching, adjacent non-cancerous tissues (N1 and N2). Protein (25 μg) from each sample was evaluated for Oct4 expression using an anti-Oct4 polyclonal antibody (sc-8629). The expression of GAPDH was used as a loading control.

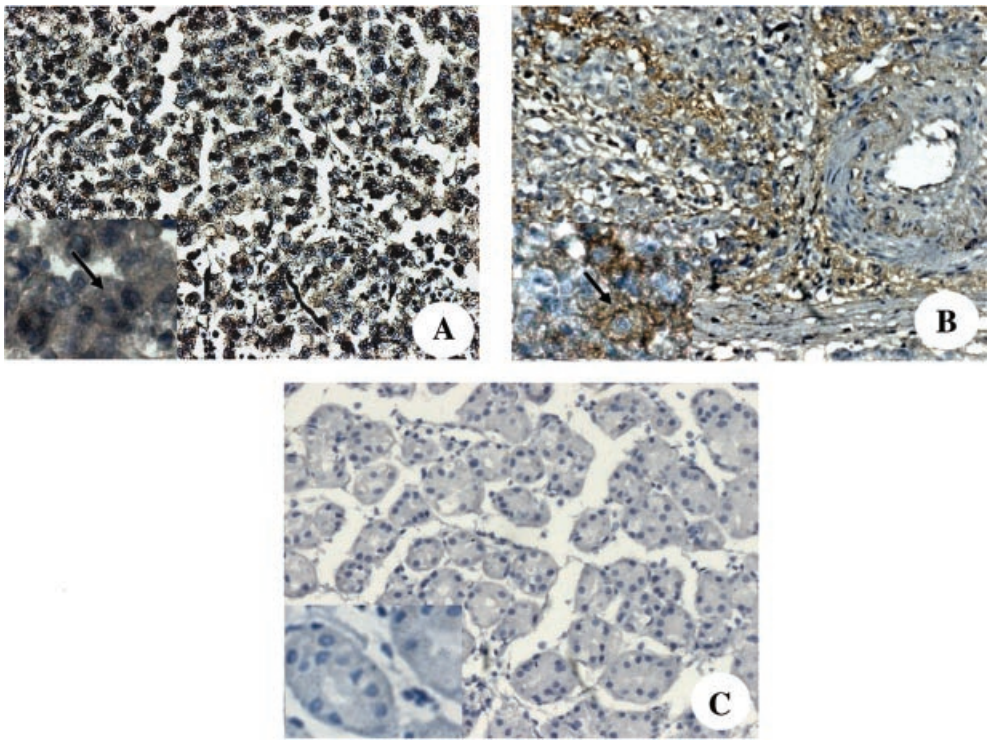

Fig. 3. Representative Oct4 protein expression, as determined by IHC, in FFPE sections of tumor and matching non-cancerous tissues. Antibodies were detected by a diaminobenzidine method that produces a brown color. Counterstaining of nuclei was accomplished with hematoxylin (blue). **A**: A seminoma showed strong nuclear and faint cytoplasmic immunoreactivity for Oct4 (dark brown, arrow; used as a positive control; 200×). **B**: Oct4 expression was mainly localized in the nuclei and cytoplasm (brown, arrow) of GC cells (200×). **C**: The matching, adjacent non-cancerous tissue was negative for Oct4 (200×). The inside square of each photomicrograph was magnified 400 times.

TABLE II. Relationship Between Oct4 mRNA Expression and Clinicopathological Parameters in 62 Gastric Cancer Patients

		Oct4 expression		
Characteristics	n	Positive	Negative	*P*-value
Age (years)				
≥60	23	14	9	0.4100
<60	39	28	11	
Gender				
Male	38	26	12	1.0000
Female	24	16	8	
Tumor size (cm)				
≥5	37	28	9	0.1655
<5	25	14	11	
Differentiation				
WD + MD	30	16	14	0.0292*
PD + UD	32	26	6	
TNM stage				
I + II	29	17	12	0.1806
III + IV	33	25	8	
Depth of invasion				
T1 + T2	28	16	12	0.1716
T3 + T4	34	26	8	
Lymph node metastasis				
Yes	38	29	9	0.0957
No	24	13	11	

WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated, UD, undifferentiated.
*Significantly different at $P < 0.05$.

in the malignant behavior of ESCs [24]. Moreover, Oct4 inactivation resulted in regression of the malignancy, suggesting that the transcription factor is essential for maintaining the malignant phenotype, and thus for determining the oncogenic fate of ESCs [16]. In order to examine Oct4 expression at the transcriptional and translational levels, tumor and matched non-cancerous tissue samples were obtained from 62 GC patients. Our results indicate that Oct4 is highly expressed in most gastric tumors, suggesting that these tissues contain stem cells, embryonic-like stem cells, or cancer stem cells (CSCs).

CSCs possess many traits ascribed to normal stem cells [25], but are able to initiate tumor growth and sustain self-renewal, and increase the metastatic potential of the tumor [26]. These cells, which are believed to give rise to tumors and phenotypically diverse tumor cell populations [27–29], have been discovered in solid tumors (such as breast, brain, and bladder) and hematopoietic tumors, and may be present in established cancer cell lines as well [8,9,30–35]. At present, there is a growing body of evidence supporting the cancer stem cell hypothesis and the role of stem cells in carcinogenesis. Since Oct4 is a marker for stem cells, including CSCs, it is possible that Oct4 can represent a novel tumor marker [36,37].

The current paradigm driving the stem cell hypothesis is that a normal cell must first be ''immortalized'' and then neoplastically transformed [37]. This idea clearly conforms to the classical multi-step model of carcinogenesis which requires a cell to receive multiple ''hits'' before it is transformed. Since stem cells are long-lived, they are more likely to acquire these multiple ''hits'' and become transformed.

GC cells over-expressing Oct4 possess the abilities to self-renew into new stem cells with identical proliferation and to maintain their undifferentiated status. When the expression level of Oct4 is down-regulated, differentiation of GC cells occurred [48]. In agreement with our data, over-expression of Oct4 appears to contribute to the neoplastic process, as Oct4 expression correlates with the poorly differentiated and undifferentiated states of GC. We believe that elevated level of Oct4 expression helps maintain cancer cells in an undifferentiated state, thus increasing their capacity for self-renewal or proliferation. In contrast to carcinogenesis, during normal development, ESC differentiation is associated with, and potentially caused by, down-regulation of both Oct4 gene and protein expression [6,7]. Further supporting the role of Oct4 in gastric cancer initiation and progression, expression level of Oct4 was down-regulated in the moderately and well-differentiated GC tissue samples as compared with that in the poorly differentiated and undifferentiated states of GC samples.

Recent studies support the use of Oct4 as a specific and useful diagnostic tool in seminomas, embryonal carcinomas, and mixed germ-cell tumors [17,38]. Jones et al. reported greater than 90% nuclear Oct4 staining in embryonal carcinoma and seminoma cells and positive immunoreactivity in all primary testicular neoplasms investigated [17,38,39]. However, the relevance of Oct4 expression in non-germ cell tumors is not well-documented. A few studies have been conducted using human kidney and lung cancer samples [40], human breast, brain, bladder cancer and osteosarcoma biopsies [8,10,41–43], human cancer cell lines [42], oral squamous cell carcinoma [44], clear cell renal cell carcinoma, and clear cell adenocarcinoma of the ovary [17,45], but no studies had previously examined Oct4 in GC.

Over-expression of the Oct4 gene is thought to lead to inappropriate activation of growth factors, promotion of cellular proliferation, and ultimately, malignant transformation [14]. In support of this hypothesis, we observed minimal Oct4 expression in non-cancerous tissues (6.5%) compared to GC tumor tissues (67.7%). Moreover, Oct4-positive cells were not equally distributed between tumors, with heterogeneous phenotypes ranging from diffusely scattered cells to aggregated cell clusters. A similar observation, reported by Gibbs et al. [46], describes variable Oct4 expression (ranging from 1% to 25%) in bone sarcoma cells and tissue specimens. We also observed strong nuclear staining and weak cytoplasmic staining of Oct4 in GC cells, a result similar to that reported by others in ESCs [38]. It seems likely that the expression of Oct4 in gastric non-cancerous tissues may indicate the presence of rare (but non-malignant) gastric stem cells, consistent with the hypothesis that normal adult stem cells serve as targets for the induction of carcinogenesis. However, more work is needed. In particular, a correlation between Oct4 expression and the recurrence and prognosis of patients with GC after surgery, radiotherapy, and/or chemotherapy has not been established.

Although a low level of Oct4 expression in human adult tissues was previously reported [47], the significance of these results remained unclear until later studies demonstrated the expression of Oct4 mRNA and protein in several human tissue-specific adult stem cells [48] and in normal human endometrium [49]. Additionally, we had previously demonstrated that novel tumor cell lines could be derived from mutations in normal stem cells [50]. All of these findings indicate that human adult stem cells have a role in tumorigenesis, that Oct4 is expressed by stem cells, and that Oct4 may have potential as a biomarker for human cancers.

To ascertain whether Oct4 is over-expressed in atrophic gastritis or gastric ulcer disease which increase the risk for GC. Our results revealed that the positive rates of Oct4 expression in atrophic gastritis and gastric ulcer groups were markedly lower. The causal association may be related to characteristics of the gastric-specific pathogen *H. pylori* [51]. Abundant evidence demonstrates that *H. pylori* infection is the risk factor for GC [52,53].

To our knowledge, the current study is the first to establish a correlation between Oct4 expression and GC. Taken together, our findings suggest that Oct4 may function as a novel oncogene, and that its expression can be used as a biomarker for GC. That Oct4 is implicated in the de-differentiation of cells and is a marker for stem cell populations further supports the cancer stem cell hypothesis, and indicates that amplification of resident GC stem cell populations (or the reversion of normal gastric stem cells to a stem cell-like state) can results in the initiation, progression, and differentiation of human GC. Further studies are needed, but Oct4 may eventually represent a biomarker that can be used to diagnose GC, or to monitor cancer progression and response to therapy.

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