PROX1 Expression in Gastric Cancer: From Hypothesis to Evidence

OANA TABAN1, ANCA MARIA CIMPEAN1, MARIUS RAICA1 and SORIN OLARIU2

1Department of Microscopic Morphology/Histology, Angiogenesis Research Center, Timis, Romania; 2Department of Surgery I, County Hospital Timisoara “Victor Babeș” University of Medicine and Pharmacy, Timişoara, Timis, Romania

Abstract. Background: PROX1 is involved in cancer development and progression as both a tumor suppressor and oncogene. Immunohistochemical (IHC) PROX1 nuclear expression is a widely accepted pattern. Scattered data reported PROX1 IHC cytoplasmic expression in different tumors, including gastric cancer but it is not clear if this holds true. Materials and Methods: Evaluation of the cytoplasmic expression of PROX1 in normal gastric mucosa and gastric cancer was performed by IHC followed by RNAscope, an in situ hybridization-based method for detecting PROX1 mRNA amplification on paraffin-embedded samples and to evaluate its clinical impact. Results: Twenty-five out of 48 cases of gastric cancer showed PROX1 nuclear and cytoplasmic immunohistochemical expression. Twelve out of these 20 cases positive for PROX1 on IHC (54.5%) had PROX1 mRNA gene amplification. The overlapping of PROX1 cytoplasmic expression assessed by immunohistochemistry and cytoplasmic RNAscope amplification was statistically significant (p=0.031). PROX1 mRNA gene amplification correlated with tumor grade (p=0.05) and regional lymph node metastasis as well (p=0.033). No significant correlation was obtained between PROX1 and histopathology, tumor size or distal metastasis. Conclusion: A significant correlation was found between IHC and RNAscope PROX1 expression in the cytoplasm of normal and gastric cancer cells. This strongly supports its validation as a true expression on immunohistochemistry. A strong correlation between PROX1 mRNA amplification and regional lymph node metastasis supports its implications in cancer spreading and metastasis and sustains its utility, not only as a lymphatic marker, but also as a potential tumor marker in various tumor types, including gastric cancer.

Although the incidence and mortality rate of gastric cancer have significantly decreased over the past 70 years, this disease is still the fourth most frequent malignancy and the second cause of cancer-related death. In 2000, almost 888,000 patients were diagnosed with gastric cancer, and it caused over 650,000 deaths. In the European countries, the five-year survival rate of gastric cancer patients is extremely low, ranging between 10% and 20% (1). Despite the study of several prognostic and therapeutic markers by immunohistochemistry and molecular methods, none of the proposed markers can be considered to have a major clinical and therapeutic impact at the moment.

The human PROX1 gene (prospero homebox gene 1) encodes a nuclear transcription factor that controls the differentiation of the lymphatic endothelium and is expressed in the nuclei of committed endothelial cells through a lymphatic lineage. In addition, PROX1 is involved in the development of myocardial, liver and pancreatic tissues (2). Up until now, the expression of PROX1 has been intensely studied not only in lymphatic vessels, but also in various tumors, such as colon cancer (3), breast cancer (4), pancreatic cancer (5), and ovarian cancer (6), as well as in vascular tumors, like the Kaposi sarcoma and the kaposiform hemangioendothelioma (7, 8).

PROX1 assessment was evaluated by quantification of nuclear immunostaining. Scattered data reported in different tumor types (including gastric cancer) PROX1 cytoplasmic expression by immunohistochemistry (IHC) staining, but this fact is not accepted as a standard parameter for interpretation, although the same studies agreed that PROX1 is present in the cytoplasm of the cell before being translocated to the nucleus to perform its biological function, and thus a cytoplasmic staining may be a correct detection approach. It has also been demonstrated that Prospero, the Drosophila counterpart of PROX1, is often found in the cytoplasm of proliferating and undifferentiated cells (9).
Based on these evidence, we sought to validate the cytoplasmic expression of PROX1 in normal and tumor gastric mucosa as a true or false expression and also to find out if the PROX1 expression and amplification could have a clinical impact for patients with gastric cancer.

Materials and Methods

Fifty cases of gastric carcinoma were diagnosed on tissue samples and included in the present study. The patients with ages ranging between 42 and 88 years were admitted to the hospital and underwent no oncological treatment prior to surgery. Open surgery, removal of the tumors and biopsy selection was performed. Three normal gastric tissue samples were harvested during autopsy procedure from patients with other pathology. The gastric cancer tissue samples were fixed in 10% neutral-buffered formalin for 24 h. The histopathological assessment was performed on haematoxylin and eosin (H&E)-stained slides for the grading of the malignant tumors and their inclusion in one of the Lauren and WHO classification systems category. In order to assess the stage of the disease, the classification recommended by the American Joint Committee on Cancer (AJCC) was used. Based on the H&E assessment of the slides, we selected sections for immunohistochemistry and the RNAscope in situ hybridization method. The immunohistochemical reactions for PROX1 were performed in all 50 cases, from which we selected 20 of them for the RNAscope technique.

Immunohistochemistry. PROX1 expression was assessed on paraffin-embedded tissues that were cut into 4-μm thick sections. The sections were then de-waxed in benzene and rehydrated through graded alcohol series. For antigen retrieval, the sections were automatically heated in the PT-LINK Pretreatment Module of the Autostainer (Dako) using citrate buffer, pH6 for 30 min at 99°C. The immunohistochemical staining was automatically performed with DakoCytomation Autostainer (Dako, Carpinteria USA). Briefly, after the hydrogen peroxide blocking step, the slides were incubated with anti-PROX1 antibody (rabbit polyclonal, code 102-PABi32, Realitech, Wolfenbüttel, Germany, Europe) for 30 min followed by detection with NOVOLINK Polymer Detection System Kit HRP (Leica Microsystem, UK, Europe). The final product of the reaction was visualized with 3,3’ diaminobenzidine chromogen as a brown staining. We used as a positive control the staining of the lymphatic compartment of the cells from the normal gastric glands, as a positive signal in both the nuclear and cytoplasmic compartment. The highest intensity was assessed as +3 (intense) for the nuclear pattern and +2 (moderate) for the cytoplasmic component (Figure 1b). When we performed RNAscope analysis on the normal gastric mucosa, we detected the presence of PROX1 mRNA as a positive signal in both the nuclear and cytoplasmic compartment of the cells from the normal gastric glands, highlighted as 3 to 5 brown dots/cell (Figure 1c). Also, for isolated cells from the normal gastric glands, PROX1 mRNA was quantified as low amplification (scored as +2) by the presence of more than 5 dots/ cell (Figure 1d).

RNAscope in situ hybridization technique. The slides were de-waxed and treated with 100% alcohol followed by air-drying. Then, using protocol of RNAscope 2.0 FFPE Brown Reagent Kit from ACDBio Advanced Cell Diagnostic (Hayward, CA 94545, USA) processed the specimens. We applied the Pretreat1 solution at room temperature for 10 min, followed by 15 min Pretreat 2 at 99°C and Pretreat3 30 min at 400C, for tissue permeabilization. The pre-warmed PROX1 mRNA probe was applied and tissues were incubated with it at 40°C, for 2 h. Signal amplification was obtained by consecutive applications of six amplifiers at 40°C (Amp1, Amp2, Amp3) and at room temperature (Amp4, Amp5, Amp6). Signal detection was performed with special mixing solution of diaminobenzidine applied for 10 min on the tissue specimens. Nuclear counterstain followed the RNAscope procedure and was done by using modified Lile’ haematoxylin (DAKO, Carpinteria, USA).

Microscopic analysis and image acquisition. These processes were performed by three different trained pathologists by using Nikon Eclipse E600 Microscope equipped with Nikon Photo Camera. The immunohistochemical staining was evaluated according to its intensity in strong (+3), moderate (+2), weak (+1 ) or none (0) and according to its localization in the cells: nuclear, cytoplasmic or both. PROX1 mRNA amplification was assessed according to the criteria recommended by the manufacturer as it is presented in Table 1.

Statistical analysis. Analysis was performed using the SPSS software, version 17. The statistical methods included three correlation tests (Spearman, Kendall and Pearson tests) applied for correlation of the immunohistochemical and in situ hybridization results together with histopathology, tumor grade, stage and lymph node metastatic status. A significant correlation was considered if p<0.05.

Results

Through the histopathological evaluation of the gastric cancer tissues on H&E-stained slides, we identified 11 cases of signet ring cell/diffuse type carcinomas, 31 cases of intestinal-type gastric carcinomas and 8 cases of indeterminate tumors, according to the Lauren classification system.

The normal gastric mucosa adjacent to the gastric tumors from our samples or harvested during autopsy had an intense immunohistochemical positive reaction for PROX1 showing both a nuclear and cytoplasmic pattern. The highest intensity was observed as to be located in the basal portion of the gastric glands (Figure 1a). Immunohistochemical staining was assessed as +3 (intense) for the nuclear pattern and +2 (moderate) for the cytoplasmic component (Figure 1b). When we performed RNAscope analysis on the normal gastric mucosa, we detected the presence of PROX1 mRNA as a positive signal in both the nuclear and cytoplasmic compartment of the cells from the normal gastric glands, highlighted as 3 to 5 brown dots/cell (Figure 1c). Also, for isolated cells from the normal gastric glands, PROX1 mRNA was quantified as low amplification (scored as +2) by the presence of more than 5 dots/ cell (Figure 1d).

PROX1 immunoreactivity gradually decreased by passing from the normal tissue to the dysplastic areas (Figure 1a). PROX1 immunohistochemical expression in gastric cancer showed a high heterogeneity concerning its location and intensity score. Ten out of 50 cases were negative for PROX1 as assessed by immunohistochemistry (Figure 2a), 14 had a weak reaction scored as +1 (Figure 2b), 11 showed a moderate intensity (+2) (Figure 2c) and other 15 were intensely stained (+3) for PROX1 (Figure 2d). Only one case presented a immunohistochemical expression of PROX1 restricted to the nucleus, 17 cases had a cytoplasmic expression exclusively and, for 22 cases there was both a nuclear and cytoplasmic IHC expression.
Despite the fact that the most intestinal type gastric carcinomas included in the study had an intense immunohistochemical PROX1 expression noted as +3 with both a cytoplasmic and nuclear pattern, a significant correlation between histopathology and immunohistochemistry was not found.

Twelve out of the 20 cases of gastric cancer (60%) assessed by RNAscope showed significant amplification for PROX1 mRNA, scored as 2, 3 and 4 according to the interpretation guidelines (Figure 2). Despite the high percent of cases showing PROX1 mRNA amplification, we observed a significant variability concerning the percent of tumor cells showing amplification and also, a distribution heterogeneity of these cells from a tumor area to another for the same case.

A particular aspect of PROX1 IHC expression and PROX1 mRNA amplification was observed in the perineural and lymphatic intravascular invasion areas. Invasive tumor areas and tumor emboli were intensely positive for PROX1 IHC and some cases presented PROX1 mRNA amplification with no positivity for PROX1 on immunohistochemistry or had a higher intensity for tumors with weak or moderate expression of PROX1 (Figure 3).

Statistical analysis was performed to detect the overlapping of the cytoplasmic IHC expression and mRNA amplification in the tumor cells. We found a significant correlation between the cytoplasmic expression of PROX1 assessed by immunohistochemistry and mRNA amplification \(p=0.031\) (Figure 4). This strongly supports the fact that PROX1 IHC cytoplasmic expression is not false-positive for PROX1 in gastric cancer and should be taken into consideration for evaluation.

**Discussion**

PROX1 is a forkhead-box-transcription factor. The protein encoded by the PROX1 gene is a member of the homebox transcription factor family. It has been shown to act as a key regulator in early embryonic development and to be implicated in the development of lymphatic vessels, the specification of horizontal cells in the mouse retina, the differentiation of hepatoblasts into hepatocytes (4) and the development of the central nervous system. Alterations of the PROX1 gene, in both its expression and function, have been linked to a large number of malignancies such as carcinomas of the pancreas, liver and the biliary duct, breast cancer, kaposiform hemangioendothelioma, colon cancer, brain tumors and various hematological malignancies. Although it has been proven that PROX1 is involved in cancer progression and metastasis, the exact mechanism through which it controls proliferation, migration and invasion ability of cancer cells, is largely unknown (10).

Several studies have evaluated the PROX1 prognostic and therapeutic value. A study performed on 56 paraffin-embedded astrocytic brain tumors showed that the number of PROX1-positive cells was correlated to high tumor grade and concluded that PROX1 can be used as a diagnostic tool to distinguish between grade III, IV tumors and grade II astrocytomas. This finding led researchers to investigate PROX1’s prognostic and predictive value in grade II tumors. Therefore, another study was conducted, on 128 cases of grade II astrocytic brain tumors that proved that PROX1 is a highly dependant predictive factor for short-term survival in patients with astrocytic gliomas, but not for those with oligodendrogliomas (11, 12).

Another research group evaluated the prognostic impact of the immunohistochemical expression of PROX1 in 517 cases of colorectal cancer. They reported that high PROX1 expression was associated with a poor grade of tumor differentiation \(p<0.0001\) and, in the case of patients with colon cancer, an unfavorable colorectal cancer-specific survival rate as compared with low PROX1 expression. (CCSS 47% vs. 62%; \(p=0.045\); RR 1.47) (13). No similar data are available for gastric cancer. We found a significant
Figure 1. PROX immunohistochemical expression in normal gastric mucosa and dysplastic adjacent tissue (a). Note the gradual loss of PROX1 immunohistochemical expression from normal gastric glands to dysplastic lesion. Detail from the basal portion of the normal gastric glands showing an intense IHC PROX1 expression (b) with both nuclear and cytoplasmic pattern (b, inset). PROX1 mRNA expression in normal gastric mucosa, showing the presence but not the amplification of PROX1 gene in the normal gastric glands cells (c) and isolated normal cells with a moderate amplification of PROX1 (assessed by the presence of more than 5 dots/cell (d).

Figure 2. Lymphatic tumor emboli intensely stained for PROX1 by immunohistochemistry compared to negative tumor cells (upper left quadrant) (a). PROX1 overexpression was confirmed by RNAscope amplification in the same area (a). Perineural invasion of gastric cancer tumor cells was accompanied by a high expression of PROX1 (b) confirmed as true by PROX1 mRNA amplification scored as 4 (b).
correlation between PROX1 mRNA amplification and histological tumor grade in gastric cancer. Our results showed that gastric tumors graded as G2 (moderately differentiated), especially those classified as intestinal-type gastric carcinomas expressed significantly higher levels of PROX1 in tumor cells assessed by both immuno-histochemistry and RNAscope method. Another study assessing the relationship between PROX1 expression in 65 patients that suffered from ovarian cancer and the main histological types, differentiation of the tumor cells and FIGO stage, failed to produce results of statistical significance (p>0.05) (6).

Patients with hepatocellular or metastatic colorectal carcinomas have lymphatic vessels, that expressed both LYVE-1 and PROX1 by double-immunostaining, only in the peripheral tumor area, thereby showing that intratumoral lymphangiogenesis is not compulsory for lymphatic metastasis of the primary tumors (14). A different experiment performed on animal subjects confirmed that PROX1 is able to suppress the proliferation of hepatocellular carcinomas by inhibiting Twist to trigger p53-dependent senescence-like phenotype (15).

Recent studies performed on a series of follicular thyroid cancer (FTC) and papillary thyroid cancer (PTC) cell lines suggested that PROX1 holds a leading role in the invasion and metastasis of these subtypes of carcinoma (16). High levels of PROX1 mRNA expressed by tumor cells invading perineural tissue or by the intravascular tumor emboli as we observed in different types of gastric cancer, support the involvement of PROX1 as one of the main promoters of tumor invasion and metastasis in gastric cancer and suggest its assessment as prognostic factor for such malignancies.

The findings of a recent study suggest that the RNA mutation of the PROX1 gene plays a major role in human cancer progression. By suffering an adenosine-to-inosine nucleotide conversion, it loses its tumor-suppressive function in a subset of human cancers, e.g., in the case of pancreatic cancer, where the expression of PROX1 is reduced (17). Also, authors showed that in the case of neuroblastomas, PROX1 contributes to the tumor’s progression and lymphatic spread, while the lymphatic density in the tumor can be correlated with the lymph node metastasis (18, 19). We also demonstrated by immunohistochemistry and RNAscope assessment PROX1 overexpression in gastric cancer cases with regional lymph node metastasis together with a high positivity for PROX1 in the tumor emboli inside peritumoral lymphatic vessels. These findings support PROX1 to be a marker for tumor aggressiveness.

A microarray-based genome-wide methylation analysis of sporadic breast carcinomas identified a hyper-methylated CpG island within the first intron of the PROX1 gene,
Figure 6. Comparative expression of PROX1 in gastric cancer assessed by immunohistochemistry and the RNAscope method. Negative immunohistochemistry overlapped with no amplification for PROX1 (a, e). Weak immunohistochemical expression for PROX1 (b) was confirmed by a low RNAscope amplification score (f). Moderate PROX1 immunohistochemical expression (c) had a score of 3 by RNAscope (g) and an intense IHC expression both nuclear and cytoplasmic was scored as 4 by RNAscope assessment (h).
making it a novel target gene that appears to be hypermethylated and transcriptionally-silenced in primary and metastatic breast cancer (4).

The cytoplasmic expression of PROX1 is a very controversial issue. Although it has been observed on IHC, it is not yet accepted as a standard parameter and needs further validation using other methods. Certain studies reported PROX1 cytoplasmic expression by IHC staining in different tumor types, as in the case of colorectal cancer. The same study admits that there is a reason for the cytoplasmic localization of the expression, because PROX1 is enriched and activated in the cytoplasm of the cell before being translocated to the nucleus to become functionally-active (13). Additionally, it has been proven that Prospero, the homolog of PROX1, translocated to the nucleus to become functionally-active and activated in the cytoplasm of the cell before being translocated to the nucleus to become functionally-active (13). Additionally, it has been proven that Prospero, the Drosophila counterpart of PROX1, is usually found in the cytoplasm of young proliferating cells (16).

Another study examined the expression of Podoplanin and PROX1 in the PTC and FTC cell lines and a series of differentiated thyroid cancer using quantitative real-time PCR (Q-RT-PCR), western blot and immunochemical methods, such as IHC and immunofluorescence. Podoplanin-negative FTC cell lines highly expressed PROX1 mRNA and in all the cell lines examined, the PROX1 protein exhibited a diffuse nucleocytoplasmic pattern. However, in the normal thyroid and peritumoral tissue, PROX1 was strongly expressed in the cytoplasm, weakly in the nuclei that lie in the peritumoral tissue, that were positive for podoplanin and clearly in nuclei of the normal thyroid (20).

In a more elaborated experiment conducted in the Yale University, ProxTom transgenic mice expressed a red fluorescent protein, tdTomato, under the direction of a PROX1 promoter. The fluorescent marker could then be identified in various sites: lymphatic vessels, adult liver, lens, dentate gyrus, the neuroendocrine cells of the adrenal medulla, megakaryocytes and platelets (21, 22). Although the insertion of tdTomato was upstream of the PROX1 nuclear localization, fluorescence was only noticed in the cytoplasm of the cells. The lymphatic vessels were co-stained with an anti-PROX1 antibody to check for transgene expression. The researchers then observed tdTomato was detectable mainly in the cytoplasm, whereas endogenous PROX1 was predominantly found in the nuclei of lymphatic endothelial cells (23).

The high-resolution confocal microscopy demonstrated that the PROX1 protein is cytoplasmic in the lens placode and in the lens epithelium or the germinative zone throughout the whole development of the lens. During fiber cell differentiation and finally, as lens fiber cells condense their chromatin, PROX1 protein is reordered and remains in the cell nuclei (22).

A similar study, that mapped the location of PROX1 in transfected Chinese Hamster Ovary cells using fluorescent microscopy observation and western blotting, reported that abundant PROX1 are found in the nucleus, but also exists scattered in the cytoplasm of the cells. The prevailing location of PROX1 is diverse and depends on the tumor cell type, as well as on the stage of the disease (23).

All these studies that investigate the expression of PROX1 and validate its cytoplasmic expression, are employing RT-PCR, PCR or western blot. Unfortunately, by adopting these methods, the cellular accuracy is lost. On the contrary, the RNAscope technique has the major advantage that the morphological structure of the tissue and the genetic expression of target molecules can be overlapped. We showed herein, that the IHC cytoplasmic expression of PROX1 correlated with its mRNA amplification as assessed by RNAscope. Through this method we were able to detect PROX1 mRNA amplification in close relationship with morphological aspect and, also to highlight the intra-tumor PROX1 heterogeneous expression.

However, there are no studies that report the expression of PROX1 in normal gastric mucosa. Our data highlighted that cells from the basal part of the gastric glands expressed PROX1 mRNA both in the nucleus and in the cytoplasm and this confirmed as true the immunohistochemical staining.

Concerning the cytoplasmic PROX1 expression in tumor cells, several articles reported a combined nuclear and cytoplasmic expression (24-26) and suggested that this dual expression is characteristic for highly-aggressive cells. Detection of both nuclear and cytoplasmic expression pattern mainly located in the invasive areas from the studied gastric carcinomas suggested the PROX1 involvement as a factor of increased aggressiveness.

**Conclusion**

PROX1 is mainly known as a lymphatic marker and little data is available regarding its involvement in tumor pathogenesis. In the case of gastric carcinomas, the overlapping results between IHC and RNAscope strongly suggest that the cytoplasmic expression of PROX1 by IHC is not a false-positive expression and impose the need to validate its expression through at least one or more methods, like RNAscope or another in situ hybridization technique. Also, in the invasion areas, in the tumor cell emboli and in the areas of peri-neural invasion, the expression of PROX1 by IHC is stronger than in the tumor, aspect confirmed by the RNAscope, suggesting that PROX1 has indeed a key role in cancer invasion and metastasis.

The fact that the expression of PROX1 in tumor cells is not only nuclear, but both nuclear and cytoplasmic and that PROX1 is strongly expressed in the invasion areas and in the tumor emboli, brings us one step closer to understanding its implications in cancer spreading. In fact, our research brings further proof that PROX1 holds a key role in cancer invasion and metastasis and supports its utility, not only as a lymphatic marker, but also as a potential tumor marker in various tumor types, especially in gastric cancer.
Acknowledgements

All Authors have an equal contribution to the present work and can be considered all as principal authors. No conflict of interests declared.

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