RESEARCH COMMUNICATION

Endo-sulfatase Sulf-1 Protein Expression is Down-regulated in Gastric Cancer

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Abstract

In our recent report on gene expression in gastric cancer we identified the endo-sulfatase Sulf-1 gene to be up-regulated in gastric tumors relative to apparently normal (AN), and paired normal (PN) gastric tissue samples. In the present report we investigate the protein expression levels of Sulf-1 gene in gastric tumors, AN and PN samples using tissue microarray (TMA) and immunohistochemistry. Expression data was collected from two sets of TMA's containing replicate sections of tissue samples. Scoring data from TMA set-1 revealed a significant difference in Sulf-1 immunoreactivity between tumors and "normals" (PN and AN) (p-value = 0.001928). Also, Sulf-1 expression in tumors was also significantly different from either PN (p-value =0.019) or AN (p-value = 0.006) samples. Similar results were obtained from analysis of scoring data from the second set of arrays. Comparison of mRNA expression and protein expression in gastric tumor tissues revealed that in 6/20 (30%) tumor samples showed up-regulated protein expression concordant with over-expression of mRNA. However, a discord with mRNA being over-expressed relative to down regulated protein expression was observed in majority 14/20 (70%) of tumor samples. Our study indicates down regulation of Sulf-1 protein expression in gastric tumors relative to PN and AN samples which is discordant with mRNA over-expression seen in tumors.

Keywords: Gastric cancer - endo-sulfatase Sulf-1 - gene expression - protein level - discordant findings

Asian Pacific J Cancer Prev, **13**, 641-646

Introduction

Gastric cancer is one of the common cancers seen in South India. Unfortunately more than 90% of patients are in advanced stage of the disease by the time they report to a tertiary centre and their prognosis is dismal. In view of highly aggressive and lethal nature of the disease there is an urgent need to characterize these tumors and identify potential biomarkers which can aid in the early detection and diagnosis. The human Sulf-1 gene codes for an endo-sulfatase with specificity for Heparan sulfate. The Sulf-1 enzyme exists in a cell surface-associated soluble form and hydrolyzes the 6-O sulfate of heparan sulfate proteogalycans (HSPGs) (Morimoto-Tomita et al., 2002). Sulf1 can enzymatically alter the HSPGs on the cell surface and regulate the activity of FGF (Lai et al., 2004a), HGF (Lai et al., 2004b), VEGF (Narita et al., 2006). Sulf-1 and another endosulfatase with similar activity, namely Sulf-2 are very similar in structural organization but are also divergent in sequence identity (64%) in humans. Analysis of human tumor derived cell lines indicate that the Sulf-1 gene is down-regulated in ovarian, breast (Chen et al., 2009; Narita et al., 2007), pancreatic, renal (Lai et al., 2003) and hepatocellular carcinoma (Lai et al., 2008). There is evidence which indicate that re-expression of hSulf-1 inhibits tumor cell proliferation, motility and invasion, HGF signaling and enhancing drug induced apoptosis in cells originating from squamous cell carcinomas of the head and neck (Lai et al., 2004a), ovarian cancer (Lai et al., 2003). On the contrary a report on the role of extracellular sulfatases in pancreatic cancer cells along with elements of the Wnt signaling pathway indicate Sulfs can enhance Wnt signaling in pancreatic cancer cells and contribute to the growth and tumorigenicity of these cells (Nawroth et al., 2007). Results from analysis in surgical tumor tissues obtained from cancer patients indicate that in some tumors protein expression of Sulf-1 is up-regulated and while in others it is down regulated relative to the normal. A study in gastric cancer identified Sulf-1 gene expression to be down regulated in gastric cancer cell lines and that DNA methylation was involved in the down regulation (Chen et al., 2009).

Our micro-array analysis of gastric tumors, paired normal (PN), and apparently normals (AN) had shown that the mRNA levels of the gene encoding Sulf-1 was up-regulated in tumors relative to AN and PN gastric tissue sample. Subsequently this result was validated by real-time PCR analysis for the expression of Sulf-1(Rajkumar et al., 2010). Similarly another report on gene expression analysis in gastric cancer also indentified that Sulf-1 was over-expressed in gastric tumors compared to

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normal tissue (Junnila et al., 2010). These observations leave open the question on the role of the Sulf-1 gene in tumorigenesis and progression of gastric cancer. In the present study we report on the protein expression pattern of Sulf-1 gene in gastric tumors, AN, and PN gastric tissue samples arrayed on a tissue microarray (TMA) using immunohistochemistry.

Materials and Methods

Gastric tissue samples

For the study 96 gastric samples were taken up for analysis using tissue microarray. The sample number comprised of 53 gastric tumor samples, 24 apparently normal, and 19 paired normal samples. The gastric cancer patient characteristics are summarized in Table1. The criteria for choosing AN, and PN was based on our previous report on gastric cancer (Rajkumar et al., 2010). Briefly, apparently normal gastric tissues were obtained from patients who were admitted for treatment for other malignancies and underwent stomach resection as part of their primary surgery and did not exhibit any lesions in the stomach and paired normal gastric tissue samples were obtained away from the resected margins of the tumor. All normal samples were hematoxilin and eosin (HE) stained and confirmed for presence of cytologically normal cells and absence of tumor cells.

Tissue microarray

The corresponding HE stained sections of each of the paraffin embedded tissue blocks were reviewed for the representative nature of the tissue samples (normal or tumor) before selection of the sample for arraying. Two 1.0 mm core from the selected tissue blocks was taken and embedded into two separate paraffin blocks. The block containing the cores of the tissue samples was sectioned to yield 0.5 micron thick sections which were transferred on to APES coated slides. Each of the arrays was re-stained with HE to confirm the representative nature of the sections on the array. For the study 4 arrays were prepared and designated TMA1 and TMA2--- TMA set-1; TMA3 and TMA4---TMA set-2. TMA1 and TMA3 contained samples from one group of tissue cores, and TMA2 and TMA4 contained sections from another group of tissue cores. In total each array contained 54 samples that also included samples from normal liver tissue which served as controls and landmarks in the array.

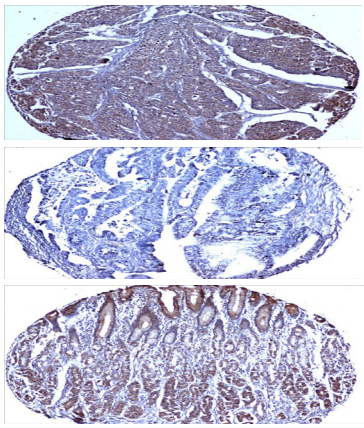

Figure 1. Representative Images from TMA Analysis of Expression of Sulf-1 in Gastric Apparent Normal, and Tumor Tissues. All the images are presented at 10X magnification. a) Sulf-1 expression in gastric tumor tissue; b) Sulf-1 non expressing gastric tumor tissue; c) Sulf-1 expression in gastric apparently normal tissue.

Immunohistochemistry (IHC)

IHC analysis for Sulf-1 expression in TMA was performed using affinity purified polyclonal antibody raised against the Sulf-1 procured from Santacruz Biotech, CA, USA (Cat no: sc-98325). 0.5 micron sections of the normal gastric tissue was used as a positive control for staining along with the TMA slides and a tissue section without the primary antibody was used as negative control. The antigen retrieval was performed by wet autoclaving method in the presence of citrate buffer pH 6.0. After the pressure in the autoclave reached 15lb/inch^2 the pressure was gradually released. Following antigen retrieval the sections were cooled to room temperature, after blocking in 2% BSA-PBS the sections were incubated overnight in primary antibody prepared at a dilution of 1:200 in blocking buffer in a humidified chamber at room temperature. The sections were stained using Super sensitive™ polymer HRP IHC detection system purchased from Bio Genex (CA, USA) as described in the protocol. The scoring of TMA was performed as previously described, briefly immunostained sections were scored by SS and TR independently and where discordant, jointly. The scoring was based on percentage of tumor cells immunoreactive (negative – 0; <25% = +1; 25-50% -+ 2; 51 – 75% + 3; >75% + 4) and intensity of immunoreactivity (negative – 0; + - 1; ++ - 2; +++ - 3). The samples were graded based on overall degree of immuno-reactivity. A score for the degree of immuno-reactivity was obtained by adding the scores for the percentage of cells expressing SULF1 and the intensity of staining. Negative immuno- reactivity in a sample was given a score of 0. Samples with a score ranging from 1 to 3 were considered as having low immunoreactivity, a score ranging from 4 to 5 as moderate or intermediate immunoreactivity and a score ranging from 6 to 7 was considered as high immuno-reactivity.

Statistical analysis

Statistical analyses were performed using OpenEpi: Open Source Epidemiologic Statistics for Public Health (www.OpenEpi.com). Fishers exact test was used test for contingency between Sulf1 expression in gastric "normal" and tumor samples. mRNA and protein expression levels in the corresponding samples were compared using matched pair study and significance assessed by Fisher exact. All results were considered statistically significant when two sided p-value was less than 0.05.

Results

Based on our gene expression analysis in gastric cancer which identified Sulf-1 mRNA levels to be up-regulated in gastric tumors relative to AN and PN, we wanted to study the protein expression of Sulf-1 gene in gastric tumors and

Table 1. SULF-1 Immunoreactivity in Gastric Tumors Data From the TMA Set-1

S.No	Patient ID	Age	HPE	Grade	IHC Score	S.No	Patient ID	Age	HPE	Grade	IHC Score
1	2688/08		AN		7	45	72/02	35 M	Adenocarcinoma	2	2
2	5711/08		AN		6	46	23/02	55 M	Adenocarcinoma	2	0
3	6518/08		AN		4	47	82/07	58 M	Adenocarcinoma	2	0
4	6776/08		AN		6	48	12/07	54 M	Adenocarcinoma	2	2
5	5991/08		AN		0	49	11/07	42 M	Adenocarcinoma	2	7
6	618/08		AN		3	50	12/08	40 M	Adenocarcinoma	2	5
7	5545/08		AN		3	51	01/01	51 M	Adenocarcinoma	3	5
8	2969/10		AN		4	52	02/01	40 M	Adenocarcinoma	3	2
9	2212/08		AN		5	53	09/01	45 F	Adenocarcinoma	3	4
10	347/08		AN		4	54	10/01	55 M	Adenocarcinoma	3	3
11	1276/09		AN		3	55	16/01	60 M	Adenocarcinoma	3	0
12	1671/09		AN		3	56	35/01	42 F	Adenocarcinoma	3	0
13	2731/09		AN		5	57	50/01	60 M	Adenocarcinoma	3	2
14	6871/09		AN		2	58	53/01	60 M	Adenocarcinoma	3	5
15	5993/09		AN		5	59	57/01	30 F	Adenocarcinoma	3	0
16	372/10		AN		7	60	62/01	57 F	Adenocarcinoma	3	0
17	510/08		AN		6	61	12/02	50 M	Adenocarcinoma	3	5
18	7871/09		AN		4	62	81/02	45 M	Adenocarcinoma	3	0
19	7250/09		AN		0	63	82/02	76 M	Adenocarcinoma	3	0
20	7625/08		AN		3	64	91/02	72 M	Adenocarcinoma	3	0
21	7506/09		AN		3	65	95/02	45 M	Adenocarcinoma	3	5
22	112/07		PN		4	66	57/02	68 M	Adenocarcinoma	3	3
23	03/08		PN		6	67	92/02	55 M	Adenocarcinoma	3	2
24	09/08		PN		2	68	62/02	51 M	Adenocarcinoma	3	2
25	42/07		PN		5	69	24/02	40 M	Adenocarcinoma	3	2
26	11/07		PN		7	70	88/07	45 F	Adenocarcinoma	3	4
27	110/07		PN		3	71	50/07	57 M	Adenocarcinoma	3	0
28	10/07		PN		4	72	98/07	50 F	Adenocarcinoma	3	0
29	48/07		PN		0	73	101/07	68 F	Adenocarcinoma	3	0
30	123/07		PN		4	74	67/07	55 F	Adenocarcinoma	3	4
31	66/07		PN		3	75	1/07	43 M	Adenocarcinoma	3	4
32	55/07		PN		4	76	43/07	55 M	Adenocarcinoma	3	2
33	88/07		PN		6	77	42/07	65 M	Adenocarcinoma	3	5
34	50/07		PN		5	78	73/07	36 M	Adenocarcinoma	3	0
35	82/07		PN		6	79	76/07	67 M	Adenocarcinoma	3	0
36	101/07		PN		4	80	123/06	56 M	Adenocarcinoma	3	3
37	98/07		PN		5	81	03/08	48 F	Adenocarcinoma	3	6
38	67/07		PN		5	82	63/06	60 M	Adenocarcinoma	3	3
39	08/01	49 M	Adenocarcinoma	2	0	83	09/08	63 M	Adenocarcinoma	3	5
40	36/01	60 M	Adenocarcinoma	2	3	84	10/07	38 F	Adenocarcinoma	3	3
41	49/01	55 F	Adenocarcinoma	2	5	85	110/07	72 M	Adenocarcinoma	3	5
42	45/02	31 M	Adenocarcinoma	2	4	86	48/07	60 M	Adenocarcinoma	3	3
43	59/02	70 F	Adenocarcinoma	2	0	87	66/07	60 M	Adenocarcinoma	3	0
44	09/02	60 M	Adenocarcinoma	2	0	88	55/07	41 M	Adenocarcinoma	3	0

normal tissue. To this end we prepared TMA's comprising of paraffin embedded gastric tumor samples (n = 53) along with AN (n = 24) and PN (n = 19) samples. The samples included 24 tumors, 4-AN and 19-PN samples in which Sulf-1 mRNA levels were previously estimated. While performing the IHC protocol some of the sections were lost or folded and hence could not be evaluated the corresponding cases were removed from the analysis. The scoring of Sulf-1 was performed blinded to the knowledge of clinical details of the samples. After staining and scoring the slides data from 50 tumor cases, and 38 "normals" comprising of both AN and PN from TMA set-1, and 47 tumors and 41 "normals" from TMA set-2 was taken up for analysis, the results of the scoring for TMA set- 1 are summarized in Table 1. The positive immunoreactivity for Sulf-1 in TMA set- 1 (Figure 1a) for tumors was 31/50 (62%) and "normals" 35/38 (92.2%) (Figure 1c), the negative immunoreactivity for Sulf-1 (Figure 1b) observed in tumors was 19/50 (38%) and "normals" was 3/38 (7.8%). The difference in immunoreactivity between tumor and "normals" was statistically significant (Table 2). The data from TMA set-2 (data not shown) also showed similar significant difference between tumors and "normals" indicating consistency in the staining pattern. When staining in tumors was compared with PN (16/17-- positive, 1/17-- negative) or AN (19/21-- positive, 2/21-- negative) the differences were significant for both PN (p-value = 0.019) and AN (p-value = 0.006).

There were no significant differences in the staining pattern observed in PN and AN samples. Similar results were also observed in data from TMA set-2. There was no correlation with other clinical parameters like grade, stage and nodal status. In the present series of samples survival analysis did not reveal significance for overall

Table 2. Comparison of Sulf-1 mRNA Overexpression (OE) and Immunoreactivity in Gastric Tumours, AN and PN from TMA Set-1

S.No	ID	Fold Change in expression relative to AN	mRNA Expression	SULF-1 Immunoreactivity	% Cells Expressing	IHC Score	Protein Expression
1	88/07/	7.25	OE	1+	3+	4	
2	50/07/	14.04	OE	Neg	0	0	
3	98/07/	10.85	OE	Neg	0	0	
4	82/07/	10.85	OE	Neg	0	0	
5	12/07/	10.85	OE	1+	1	2	
6	12/08/	34.52	OE	3+	3+	6	OE
7	11/07/	53.38	OE	3+	4+	7	OE
8	101/07/	10.85	OE	Neg	0	0	
9	67/07/	10.85	OE	1+	3+	4	
10	1/07/	10.85	OE	1+	3+	4	
11	43/07/	12.04	OE	1+	1+	2	
12	42/07/	0.96		1+	3+	4	
13	73/07	26.60	OE	Neg	0	0	
14	76/07/	8.70	OE	Neg	0	0	
15	123/06	14.55	OE	1+	2+	3	
16	03/08/	17.75	OE	3+	4+	7	OE
17	63/06/	1.14		1+	2+	3	
18	09/08/	0.40		2+	3+	5	OE
19	10/07/	15.81	OE	1+	2+	3	
20	110/07/	5.82	OE	2+	3+	5	OE
21	48/07/	2.72	OE	1+	2+	3	
22	66/07/	39.39	OE	Neg	0	0	
23	55/07	11.07	OE	Neg	0	0	
24	03/08 PN	0.74		3+	3+	6	OE
25	09/08 PN	2.03		1+	1+	2	
26	43/07 PN	1.32		ND	ND		
27	42/07 PN	1.01		2+	3+	5	OE
28	11/07 PN	1.92		3+	4+	7	OE
29	110/07 N	0.34		1+	2+	3	
30	10/07 PN	3.64	OE	1+	3+	4	
31	48/07 PN	0.46		Neg	Neg	0	
32	123/07 PN	0.98		2+	2+	4	
33	66/07 PN	1.53		2+	1+	3	
34	55/07 PN	0.87		1+	3+	4	
35	76/07 PN	0.94		ND	ND		
36	88/07 PN	1.16		2+	4+	6	OE
37	50/07 PN	1.36		2+	3+	5	OE
38	82/07 PN	0.85		3+	3+	6	OE
39	101/07 PN	2.08		2+	2+	4	
40	98/07 PN	0.94		1+	4+	5	OE
41	67/07 PN	0.88		2+	3+	5	OE

survival or disease free survival with Sulf-1 expression.

We then compared the mRNA expression levels previously estimated in the gastric tumor tissue samples (n= 23) which were also included in the TMA. The fold changes in mRNA expression of Sulf-1 for the tumor samples and PN samples relative to AN samples were retrieved from our earlier study. The details of fold changes in mRNA expression in tumors and PN and corresponding protein expression for individual samples are summarized in supplementary table 2. The details of comparison of mRNA expression with protein expression for data derived from TMA set-1 for 23 samples (1 sample was lost during the procedure and hence not included in the analysis) are summarized in table 2. In the case of mRNA expression greater than 2 fold increase or decrease relative to the expression level in AN sample was considered as over-expression or down regulation respectively. The mRNA levels showed over expression in 20/23 (87%) and less than 2-fold increase in 3/23 (13%) in tumor tissue samples none of the tumor samples indicated mRNA down regulation relative to AN. In the case of protein expression, median of the overall immunoreactivity scores from all AN samples (n=21) which was determined to be a score of 4 (range = 0-7) was taken as the cut off score. Values above the cutoff were considered as over-expression. The protein expression of Sulf-1 determined by IHC indicated that among the tumor samples 5/23 (21.7%) showed over expression and 18/23 (78.2%) did not show over-expression or indicated negative immunoreactivity. Comparison with mRNA over expression status of the samples revealed that among the 20 Sulf-1 mRNA over-expressors only 4/20 (20%) showed a concordant protein over-expression. In the case of majority 16/20 (80%) of the tumor samples protein over-expression was not observed and 8/16 (50%) of these samples showed negative immunoreactivity. The discord

between mRNA expression levels and protein expression was statistically significant (p value =0.0009766). In PN samples 1/18 samples showed over-expression and 17/18 samples showed less than 2-fold increase in mRNA expression and none of the PN samples showed down regulation. The one over-expressing sample did not indicate protein over-expression, interestingly PN samples indicating greater than 0.5 fold and <2 fold increase in RNA expression levels relative to the AN showed protein over-expression in 52% (9/17) of samples.

Discussion

Role for biomarkers in detection of early gastric cancer is of high import since early stage gastric cancer is asymptomatic and majority of the cases presenting at an advanced stage have poor prognosis. Early detection combined with accurate preoperative staging offer the best prognosis. There is currently a paucity of specific and sensitive biomarkers for early detection hence there is a need for finding specific biomarkers for diagnostic screening purposes. Routinely gastric cancers are screened by endoscopy because of a high detection rate. Population based screening programs to detect early lesions employing endoscopy in countries like Japan have enabled detection of early lesions and coincided with improved survival rates (Leung et al., 2008). However, to employ endoscopy to screen a large population as in the case of our region would be difficult to implement as the technique depends on skills of endoscopist, the availability of gastroscope and cost. Biomarkers preferably serum based capable of identifying high-risk individuals for further testing would be better suited for screening. Some serum markers already in use are Pepsinogen I and II levels, Gastrin-17 levels and H-pylori serology (Leung et al., 2008) despite their extensive use, cut off values taking into account variability in the levels based on differences in ethnicity need to determined. To identify novel biomarkers studies have employed gene expression analysis involving microarrays (Rajkumar et al., 2010; Wang et al., 2010; Yamada et al., 2008) or whole genomic sequencing analysis (Zhang et al., 2011) and proteomic analysis of gastric tissues (Bai et al., 2011; Ryu et al., 2003) and blood (Lam and Lo, 2008; Mohri et al., 2009; Qiu et al., 2009; Lu et al., 2010). Some of the potential markers reported include human neutrophil peptides 1-3 (HNPs 1-3), Macrophage migration inhibitory factor (MIF) (Mohri et al., 2009), COX-2, p53 (Mrena et al., 2010) CD44v6, MMP-7 , nuclear Cdx2 (Okayama et al., 2009) Vascular Endothelial Growth Factor-D and its receptor VEGFR-3 (Jüttner et al., 2006) require further testing on larger sample sizes to assess their validity, this combined with the fact that likely heterogeneity in the disease could arise out of ethnicity based differences, would further require one to screen and explore potential markers suitable for individuals from that region. Our results from the analysis of Sulf-1 protein expression in gastric cancer revealed significant down regulation in the tumors relative to PN and AN. The difference in expression pattern could indicate a role for Sulf-1 protein expression as a potential bio-marker for gastric cancer whose lack of expression indicates a tumorigenic phenotype.

When we compared the mRNA expression levels and corresponding protein expression in both tumors and PN relative to AN the analysis indicated negative/lower expression of Sulf-1 despite a corresponding mRNA over-expression in tumor tissue relative to AN. In the PN samples being sourced from the adjacent normal region away from the tumor consistently showed mRNA expression and protein expression levels on par with AN samples hence, gastric tumor tissues plausibly harbor changes which can alter the stability of Sulf-1 mRNA or protein. The discord between the mRNA expression level and protein expression has been reported for genes in other cancers, for example in the case of basal keratin expression in breast cancer, the mRNA expression of keratin genes CK5, CK14, and CK17 was discordant with protein expression levels determined by IHC (Kordek et al., 2010). In a larger study of lung adenocarcinomas comparison of mRNA expression with protein abundance levels identified by 2D-electrophoresis indicated positive correlation in 21.4% of the 98 genes analysed with majority of the genes showing a lack of correlation (Chen et al., 2002). Since there are still questions about the extent of correlation between the expression levels of mRNA and the corresponding changes in expression levels of proteins expressed, it is likely essential to combine global gene expression analysis with protein expression analysis to gain a better understanding of relationship between the mRNA expression and protein levels in the cancer cells. In summary, our report on Sulf-1 protein expression in gastric cancer indicates a discord between mRNA and protein expression of Sulf-1 and a putative role for Sulf-1 as bio-marker for gastric cancer.

Acknowledgements

This study was supported by a grant from the Department of Science and Technology, Govt. of India.

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