CHAPTER 5

DISCUSSION
5. DISCUSSION

The objective of this chapter is to integrate all the ideas and work presented in this study in a single frame, especially to present a coherent description of the underlying intricate concepts of the work.

Adenocarcinoma of stomach is multifactorial in nature. Gastric cancer shares some putative risk factors, including diet, low socioeconomic status, age, alcohol and tobacco, nitrites and nitrates. Gastric adenocarcinoma is the second most common cause of cancer-related mortality worldwide (National Cancer Institute). Cancer of the stomach is the consistently leading site of cancer among males comparative to female. Gastric Adenocarcinoma is more prevalent in Southern India compared to Northern India (Indian council of Medical Research). This is the first study from an Indian scenario, focusing on gene expression profiling of this tumor. Globally, this is the first kind of study using a whole human genome array.

The microarray technology, made it easier to study the multiple genes across multiple samples. Though, gene expression profiling of Gastric cancer has been done by several groups around the world using different platforms, still cytotoxic therapy remains to be the standard mode of treatment for gastric cancer patients. This emphasizes the need for more studies on identification and validation of novel molecules at the molecular and protein level to discover suitable biomarkers to understand proper diagnosis, prognosis and therapy for the treatment and cure of gastric cancer.
In the Experiment 4.1, the gene expression profile of the Gastric adenocarcinoma was studied, by doing whole human genome array using Gastric adeno carcinoma tissues and adjacent non-cancerous tissue samples from 14 different Gastric cancer patients. A fold change cut off of 4 was used to determine differentially expressed genes. Based on the above cut-off, it was observed that 232 genes were up regulated and 221 genes were down regulated in gastric cancer comparative to adjacent non cancerous tissue. Among the up-regulated genes, several genes were also reported by previous studies validating our data. Few of these genes are discussed in detail below.

Claudins belong to the family of tight junction proteins involved in maintaining cell polarity in epithelial and endothelial cells (Lal-Nag et al., 2009). In humans, 24 claudin subtypes are known among which Claudin 1, Claudin 2, Claudin 3, Claudin 4 and Claudin 6 were reported to be expressed in gastric cancer (Jung et al.; Rendon-Huerta et al.,). Claudin 1 and claudin 6 were known to be expressed more frequently in intestinal type gastric cancer as compared to diffused type (Rendon-Huerta et al.; Resnick et al., 2005). In our study claudin 1 was found to be 22 fold up-regulated and Claudin 4 and Claudin 6 were known to be 3 fold up-regulated. Osteopontin (SPP1) is a secreted phosphoprotein that belongs to the family of N linked glycoproteins and known to be expressed in epithelial tissues (Dai et al., 2007). SPP1 was known to be over expressed, (Imano et al., 2009) known to promote metastasis (Song et al., 2009) and also serves as a prognostic factor for gastric cancer (Dai et al., 2007). In this study it was found to be 14 fold up-regulated. Other markers that were significantly up-regulated and are previously known include Sulfatase 1 [SULF1] (Junnila et al.,), Inhibin, beta A INHBA (Zhang et al.,), and
High mobility group AT-hook 2 (HMGA2) (Motoyama et al., 2008) which were up-regulated 8 fold, 9 fold and 8 fold respectively

In this study it was also observed that numbers of genes were not yet described in the context of gastric cancer and are novel. Among those, some of the genes that were up-regulated are discussed in detail below.

It was found that number of genes were highly up-regulated with no known previous association to any cancer- Clarin 3 (CLRN3), Odd skipped related 2 (OSR2) and SPOCK1. CLRN3 is a trans membrane protein whose function is not yet known. OSR2 belong to the family of transcription factors and is a human homolog of drosophila odd shipped family of proteins (Lan et al., 2004). CLRN3 was found to be 12.6 fold up-regulated and OSR2 was found to be 12.9 fold up-regulated in gastric adenocarcinoma as compared to normal. It was also found that many genes were known in other cancers but not reported in gastric cancer. Chordin like 2 (CHRDL2) is an extracellular matrix protein which is also called as breast novel factor 1. It was initially identified as a novel gene over expressed in breast tumors by differential display analysis. It was also found to be over expressed in lung and colon cancers (Wu et al., 2003). In the present study CHRDL2 was 7 fold upregulated in tumor as compared to normal. Gremlin 1(GREM1) belongs to the family of BMP antagonists. It is a secreted glycoprotein known to be involved in regulation of early development (Namkoong et al., 2006). It has been reported to promote tumor cell proliferation in basal cell carcinomas (Sneddon et al., 2006) and known to be over-expressed in carcinomas of lung, ovary, uterine cervix, colon,
pancreas and sarcoma (Namkoong et al., 2006). Gremlin was 4.5 fold up-regulated in our micro array study. However in our protein level study (i.e; WB and IHC) it was found that Gremlin is down regulated in gastric cancer tissues as compared to adjacent non cancerous tissue.

Based on the results of the micro array study presented here, it reveals that the identified molecules could become clinically useful markers, if tested for their diagnostic, prognostic and therapeutic value.

In the experiment 4.2, based on the immunohistochemical analysis of Villin 1, a calcium regulated actin binding protein of intestinal brush border epithelium, it is evident that Villin 1 could be used as a biomarker for gastric adenocarcinoma. Earlier it has been reported as a marker for intestinal type gastric adeno-carcinoma (Osborn et al., 1988). It has also been shown to be expressed in gastric tubular adeno-carcinomas (Moll et al., 1987). In our microarray analysis, Villin 1 was found to be 9 fold up regulated at mRNA level.

**SPOCK1/ Testican 1**- belong to the family of calcium binding extracellular proteoglycans. Due to its modular architecture it is referred to as SPOCK1 (SPARC/Osteonectin, CWCV and kazal like domains). SPOCK1 was known to be over expressed in glioblastomas (Colin et al., 2006), prostate carcinomas (Wlazlinski et al., 2007) and gastrointestinal neuro endocrine carcinomas (Leja et al., 2009). The current study presents the first report of over expression of SPOCK1 in Gastric adenocarcinoma at molecular as well as protein level.
In the Bio-informatics analysis of differentially expressed genes of Gastric cancer, the set of Genes corresponding to Extracellular Membrane receptor interaction, focal adhesion, and cell communication, were found to be significantly up-regulated while ribosome metabolism and calcium signaling pathway were significantly down regulated.

In Biological Network analysis SPP1, CLDN1, SPOCK1 and CLDN4 formed a distinguished sub-network connected through CLDN1. Based on the network analysis, the Interaction of SPOCK1 and SPP1 with the tight junction proteins CLDN1 and CLDN4, it is most evident that they may have a major role in gastric tumorigenesis.

**Gremlin**- a bone morphogenic protein (BMP) antagonist, as per the review of literature, is over expressed in many cancers. In Micro Array analysis also we observed over expression of Gremlin in Gastric cancer. However, in Western Blotting and Immunohistochemical analysis, it was found that gremlin is down regulated in Gastric cancer. Hence, Gremlin could become a specific marker for diagnosis of Gastric adeno carcinoma. This is the first report of expression profile of Gremlin with respect to Gastric adeno carcinoma.

**Sparc Like Protein 1**- mRNA is down regulated in many epithelial tumors (Bendik et al., 1998; Claeskens et al., 2000; Nelson et al., 1998; Notterman et al., 2001). In addition, SPARCL1 can act as a negative regulator of cell proliferation when over expressed in HeLa 3S cells (Claeskens et al., 2000), therefore we hypothesized that SPARCL1 might be a novel tumor suppressor gene.
In the microarray analysis SPARCL1 is down regulated in Gastric cancer. However, there was no preliminary data of Sparc Like Protein 1 expression in Gastric cancer. Hence, Western blotting as well as Immunohistochemical analysis was done with different samples of Gastric cancer and adjacent non cancerous tissues. And it was found that SPARCL1 is down regulated in Gastric adeno carcinoma in comparison to adjacent non cancerous tissue. This is the first report of expression profile of SPARCL1 in respect of Gastric adeno carcinoma.

As it was described earlier, many molecules were found in micro array analysis to be differentially expressed in Gastric cancer. However it is not possible to develop Antibodies against every molecule, because of its cost, time etc. Hence, we tried to validate few of the novel molecules by Mass spectroscopy, which were mostly plasma membrane and extra cellular proteins. In cancer metastasis plasma membrane and extra cellular proteins play a major role and as Gastric cancer is highly metastatic these molecules could become valuable tools to study the mechanism of Gastric cancer metastasis. They are discussed in detail below.

Envoplakin (EVPL)- a member of the desmosomal plaque proteins attached to desmosomal cadherin and keratin filaments. EVPL is a plasma membrane protein known to be involved in sporadic forms of oesophageal squamous cell carcinoma (OSC) (Risk et al., 1999). In our mass spectroscopy we observed a 4 fold change of EVPL in Gastric cancer. Hence, EVPL may also involve in the metastasis of gastric cancer.
Fibulin-5 (FBLN5)- A extracellular protein that modulates elastogenesis, extracellular matrix (ECM), cell motility, cell adhesion, and binds integrins (Yanagisawa et al., 2009), was down regulated in response to tumor derived factors. FBLN5 was found to inhibit metastatic colonization of the liver and lung and to suppress the activity of matrix metalloproteinase 9 (MMP9). TGF-β stimulation in normal mammary epithelial cells led to up-regulation of FBLN5, production of MMPs, and enhanced growth of FBLN5-positive tumor cells (Lee et al., 2008). These data reveal a tumor-driven Fbln5-mediated autocrine regulation of the ability of fibroblasts to invade the ECM and generate a microenvironment favorable for metastasis formation. FBLN5 also reported to be involved in several solid tumors but not reported in gastric cancer. In our study we checked the expression of FBLN5 in gastric cancer, and in our mass spectroscopy we observed 3 fold change of FBLN5 in Gastric cancer.

Hemopexin (HPX)- Extracellular Plasma protein with a high binding affinity to heme in an equimolar ratio (Muller-Eberhard, 1998). HPX belongs to acute phase protein whose synthesis is induced after an inflammatory event (Baumann and Gauldie, 1994). The hemopexin glycan marker could be a valuable complementary test to alpha-fetoprotein measurements for detection of Hepato cellular carcinoma (HCC) in patients with cirrhosis (Evi et al., 2010). In our mass spectroscopy we observed 4 fold change of HPX in Gastric cancer. Hence, additional study of its utility for diagnosis and follow-up of Gastric cancer is recommended.
Osteoglycin preprotein isoform 1- Extracellular matrix protein. In mass spectroscopy analysis we observed 3 fold change of Osteoglycin preprotein isoform 1 in Gastric cancer.

Lumican- Extracellular protein is a member of a small leucine-rich proteoglycan family and it’s over expression in human breast cancer tissues is reported to influence the growth of cancer cells. Lumican is better known as a member of the small leucine-rich repeat proteoglycans that bind collagen and modify the structure of collagen-rich connective tissues (lozzo 1998). Lumican protein synthesized by cancer cells, fibroblasts and epithelial cells with mild reactive dysplasia found adjacent to cancer cells may affect the growth of human colorectal cancer cells (Yue Ping Lu. et al.,). In the mass spectroscopy analysis also it was found to be 3 fold changes in gastric cancer. Therefore, Lumican could play similar role in gastric cancer also.

Nef associated factor 1 (Nafl)- cytoplasmic protein. Nef induces down-regulation of cell surface expression levels of CD4 (Harris 1996; Garcia and Miller 1991) and major histocompatibility complex (MHC) class I (Schwartz et al.,1996; Greenberg et al., 1998) molecules by accelerating cellular endocytosis through clathrin-coated pits (CCPs) (Piguet et al., 1998; Le Gall 1998). Naf1 over expression increased cell surface CD4 levels (Fukushi et al., 1999). We found 5 fold change of Naf1 in Gastric cancer. However, further studies are needed to address the role of Naf1 in gastric adenocarcinoma.
C14orf55- Novel cytoplasmic protein not well characterized. In our mass spectroscopy analysis 5 fold change of C14orf55 was found and again this is the first report of expression of C14orf55 in respect to Gastric adenocarcinoma.

In brief, early diagnosis of Gastric adenocarcinoma improves prognosis, but currently available biochemical markers or imaging modalities either lack adequate sensitivity or specificity. Due to the complexity of the disease, reliable biomarkers are unlikely to be identified for sensitive and specific diagnosis of Gastric adenocarcinoma. Micro array profiling experiments offer an effective alternative for identifying differentially expressed molecules as potential biomarkers. In this study, we have identified many upregulated and downregulated proteins in Gastric adenocarcinoma that may have pathological relevance as well as diagnostic potential. To our knowledge, this is the first study from an Indian scenario, focusing on gene expression profiling of Gastric adenocarcinoma. Globally, this is the first kind of study using a whole human genome array. Importantly, we were able to identify many of the proteins that have been previously reported to be upregulated in Gastric adenocarcinoma, corroborating earlier findings of differentially expressed proteins.