Chapter 5

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5.1 Validation of CNAs in PaCa cells

Genomics tools such as aCGH and next generation sequencing provided a close up glance into the genomic aberration in cancers. These genomic aberrations often lead to the CNAs i.e. amplification of oncogenes and deletion of TSGs. Identification of these genomic aberrations and subsequent functional characterization of genes located in these abnormalities is an important step towards discovery of novel therapeutic target. Many genomic aberrations were known for PaCa and characterization of few of these genomic abnormalities led to the identification of deregulated genes having a possible oncogenic and tumor suppressor function (Hahn, Schutte et al. 1996; Bashyam, Bair et al. 2005; Kwei, Bashyam et al. 2008; Kwei, Shain et al. 2011). In-spite of molecular studies and drug targets available for PaCa, the prognosis of PaCa patients has not improved for the past thirty years (Siegel, Naishadham et al. 2012). The lethality of PaCa necessitates developing novel therapeutic targets for a better treatment. Several genomic alterations, identified in PaCa cells, and yet to be functionally characterized for identification of therapeutic targets. Among these genomic abnormalities, two frequent chromosomal deletions located at 6q25.3 and 18q23, observed in PaCa cell lines and primary tumor tissues (Furukawa and Horii 2004; Heidenblad, Schoenmakers et al. 2004; Bashyam, Bair et al. 2005; Harada, Baril et al. 2007; Birnbaum, Adelaide et al. 2011) was the focus of this study. Limited information was available for the functional significance of these two deletions during pancreatic tumorigenesis. Therefore, to elucidate the functional significance of these two deletions in PaCa, the current thesis project started with the objective of identification and functional characterization of the novel chromosomal deletions in PaCa.
5.2 Chromosomal deletion at 6q25.3 harbor ARID1B, a possible TSG in PaCa

Deletion at 6q25.3 was previously known in many cancers such as colon cancer (Lassmann, Weis et al. 2007), breast cancer (Ghazani, Arneson et al. 2007), familial lung cancer (Bailey-Wilson, Amos et al. 2004) and PaCa (Furukawa and Horii 2004; Heidenblad, Schoenmakers et al. 2004; Bashyam, Bair et al. 2005; Harada, Baril et al. 2007; Birnbaum, Adelaide et al. 2011). The aCGH data analyzed with the help of CLAC gives a comparatively non-smoothened output from aCGH data for each gene locus and it is important to analyze the aCGH using a more advanced tool which can analyze the aCGH data in a more smoothened way. CghFused Lasso is one such technique developed to carryout aCGH data analysis (Tibshirani and Wang 2008). Therefore, previous aCGH data of PaCa cell lines (Bashyam, Bair et al. 2005) was tested in CghFused Lasso analysis for identification of significant DNA amplifications and deletions yielded a smoothened profile of genomic changes across the genome of PaCa cell lines (Fig 4.1.1A and B). I have identified a single annotated gene viz ARID1B in the deletion locus in UCSC genome browser database (Fig 4.1.1c). ARID1B is a DNA binding component of ATPase dependent SWI/SNF chromatin remodeling complex (Wu, Lessard et al. 2009). SWI/SNF is a multsubunit complex and several components of SWI/SNF such as SNF5, BRG1, BRM etc was proposed to be bona fide TSG in several human cancers (Wilson and Roberts 2011). Gene mutational inactivation of TSG is common for tumor progression. For example, p53 is a classical TSG found to be inactivated through gene mutation in several cancers including PaCa (Petitjean, Achatz et al. 2007; Goh, Coffill et al. 2011). I did not find any mutation in the coding region of ARID1B in the PaCa cell lines which was consistent with the studies demonstrated in PaCa cell lines (Birnbaum, Adelaide et al. 2010). However, ARID1B mutation was detected in pediatric tumor neuroblastoma (Sausen, Leary et al. 2013), breast carcinoma (Stephens, Tarpey et al. 2012) and hepatocellular carcinoma (Fujimoto, Totoki et al. 2012). Absence of mutation in PaCa cell

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lines suggested the possible epigenetic mechanism of ARID1B gene inactivation as reported for other genes including P16 which is predominantly inactivated by DNA hyper-methylation in PaCa (Hong, Park et al. 2011). Also, other SWI/SNF components were also known to be inactivated through DNA hyper-methylation. For example, BAF47 is inactivated in pediatric cancer through hyper-methylation (Reisman, Glaros et al. 2009). Interestingly, several PaCa cell lines harbor comparatively low ARID1B transcript level (Fig 4.1.4A and B) and the mechanisms for down-regulation in these cell lines are not known. Therefore, several PaCa cell lines (harboring comparatively low ARID1B transcript level) were tested for Azacytidine and TSA mediated up-regulation of ARID1B transcript level and found increase in ARID1B transcript level in those cell lines (Fig 4.1.5 A and B). The Azacytidine mediated transcription up-regulation indicated a possible DNA hyper-methylation of ARID1B causing its transcription suppression in PaCa cell lines. For example, up regulation of HLTF was observed in colon cancer cell line upon Azacytidine treatment (Motegi, Liaw et al. 2008).

Using an *in silico* analysis of the ARID1B putative promoter region, I have detected strong CpG islands and the result of bisulfite genome sequencing analysis revealed a strong hyper-methylation upstream of translation initiation codon in SW1990 PaCa cell lines and importantly the hyper-methylation was completely lost upon Azacytidine treatment (Fig 4.1.7C). Thus, It was shown to have possible suppression of ARID1B transcription in SW1990 PaCa cell line.

SWI/SNF complex is emerging as tumor suppressor complex, since several SWI/SNF components have been shown to be inactivated through mutation or reduced expression in a variety of cancers (Wilson and Roberts 2011; Shain and Pollack 2013). Recently, several SWI/SNF components were shown to have reduced level of expression in PaCa samples (Numata, Morinaga et al. 2013). Functional implication of SWI/SNF in context of tumor progression has been assessed in several studies. Ectopic expression of SWI/SNF components

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resulted in restoration of anti-tumor properties such as cell cycle arrest, apoptosis and differentiation (Dunaief, Strober et al. 1994; Wilson and Roberts 2011). SWI/SNF modify chromatin or associate with transcription factor for transcription regulation of specific set of genes during tumor progression. For example, \textit{ARID1B} functions as scaffold for E3-ubiquitin Ligase causing ubiquitination of \textit{H2B} during transcription (Li, Trojer et al. 2010). Recently, a study has shown an interaction with \textit{BRG1} and \textit{E2F6} (a transcription repressor of \textit{E2F} responsive gene in S phase of cell cycle) and suggested transcription regulation of G1/S responsive genes (Leung and Nevins 2012). An important SWI/SNF transcriptional target, the cell cycle inhibitor \textit{p16} (Kia, Gorski et al. 2008), is frequently inactivated in PaCa (Weissman and Knudsen 2009). Mechanistically, \textit{ARID1B} might play a role in growth suppression mediated through TGFβ as it was shown to interact with the \textit{SMAD2/3} complex (Xi, He et al. 2008).

SWI/SNF complex has been implicated in maintenance and differentiation of stem cells during developmental process (Wu 2012). For example, \textit{ARID1B} levels have been shown to increase during differentiation of ES cells (Kaeser, Aslanian et al. 2008; Yan, Wang et al. 2008) and ectopic expression in HeLa cells cause impaired cell growth, accumulation of cells in G0/G1 cell cycle phases and upregulation of cell cycle arrest genes such as \textit{p53} and \textit{P21} (Inoue, Giannakopoulos et al. ; Inoue, Giannakopoulos et al. 2011). EMT and cell migration are important steps during carcinogenesis. SWI/SNF component \textit{SNF5} has been shown to interact with \textit{RHOA} and enhance cell migration in malignant rhabdoid tumor (Caramel, Quignon et al. 2008). Similarly, loss of E-cadherin is characteristic of EMT and E-cadherin repression occurs through \textit{ZEB1} transcription factor (Sanchez-Tillo, Lazaro et al. 2010). \textit{ZEB1} interaction with \textit{BRG1} in colon cancer cell line implicated involvement of SWI/SNF in EMT and cell migration (Sanchez-Tillo, Lazaro et al. 2010). These results indicate a possible tumor suppressor function for SWI/SNF in general and \textit{ARID1B} in particular in several

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cancers. Interestingly, a possible inactivation of several components of SWI/SNF in PaCa including \textit{ARID1B} suggests a possible tumor suppressor role for the complex (Dal Molin, Hong et al. 2012; Shain, Giacomini et al. 2012; Numata, Morinaga et al. 2013). I observed an abrogation of tumor related characteristics upon ectopic expression of \textit{ARID1B} in MiaPaCa2 cells as evidenced through liquid and soft colony formation assay (Fig 4.2.4a). I also observed the reduction in cell migration rate in one of the stable clone (A3) as compared to empty vector stable clone (PC15). However, I could not get the similar results with other clones (A8 and PC14) (Fig 4.2.5). Phenotypic and functional variation could be possible among the stable clones of same origin. Dendritic stable clones generated from mice epidermis have been shown to possess functional and phenotypic heterogeneity (Xu, Bergstresser et al. 1995). The phenotypic effect upon ectopic expression of \textit{ARID1B} further support its possible tumor suppressor function in PaCa cell line.

Previous studies on preosteoblast cell lines revealed opposing roles for \textit{ARID1A} and \textit{ARID1B} containing SWI/SNF complexes, with the former resulting in cell cycle arrest while the latter appeared to be involved in cell cycle progression (Nagl, Wang et al. 2007). It is possible that \textit{ARID1B} may have distinct roles depending on the cellular context. The alternative DNA binding component of SWI/SNF \textit{viz. ARID1A} appears to be a specific tumor suppressor for ovarian clear cell carcinomas (Wiegand, Shah et al. 2010). The role of \textit{ARID1A} and \textit{ARID1B} in different tumor types could possibly reflect differences in their tissue specific expression.

\textit{ARID1B} mutation was documented in several cancers including ovarian clear cell carcinoma, gastric carcinoma, breast carcinoma, etc. (Shain and Pollack 2013). However, \textit{ARID1B} expression at the protein level in cancer is not known. In a recent study, on PaCa cell lines and tissues documented the endogenous expression level of \textit{ARID1B} in few PaCa cell lines (Shain, Giacomini et al. 2012). I generated ectopically expressing stable clones of \textit{ARID1B} in MiaPaCa2 using cDNA clone obtained from Kazusa DNA Research Institute (Nagase,
Ishikawa et al. 1999). The clone has functional ARID domain required for its activity (Nagase, Ishikawa et al. 1999). KIAA1235 clone has been used for determination of transcription activation and E3 ubiquitine ligase activities (Inoue, Furukawa et al. 2002; Li, Trojer et al. 2010). I could detect antitumor phenotypic effect in MiaPaCa2 cells in the stable clones. However, I was unable to detect protein expression in western blot (4.2.1b). But expression at RNA level was readily detected (4.2.1a). The exhibited anti-tumor phenotype in MiaPaCa2 could possibly be due to undetectable level of protein in western blot. Since transcription factors are expected to generate a pleiotropic effect, a low level change in expression may be expected to result in phenotypic changes. Usually, tumor cells show reduced fitness in high level of TSG expression. MiaPaCa2 cells lack BRM (also known as SMARCA2) activity and restoration of BRM in MiaPaCa2 was successful only at a very low level of expression (Rosson, Bartlett et al. 2005). In the current study, over expression of ARID1B had no significant effect on growth of MiaPaCa2 cells though the transfectants exhibited significantly reduced ability to grow in the absence of anchorage or in the presence of contact with neighboring cells (Fig. 4.2.4a). Interestingly, the tumor suppressor SNF5 exhibits reduced colony forming ability when overexpressed in breast cancer cell line (Xu, Yan et al. 2010). Similarly, ectopic expression of BAF155, a core component of the human SWI/SNF complex, in cancer cell lines, was recently shown to reduce colony formation ability without affecting apoptosis (DelBove, Rosson et al. 2011).

Since MiaPaCa2 cells do not express BRM (Rosson, Bartlett et al. 2005), my observations pertaining to the anti-oncogenic effect of ARID1B expression could perhaps be a result of action of a SWI/SNF complex harboring BRG1 and ARID1B. Interestingly, BRG1 and ARID1B have been suggested to harbor a similar expression profile (Flores-Alcantar, Gonzalez-Sandoval et al. 2011), and often localize to transcriptionally active promoters (Flores-Alcantar, Gonzalez-Sandoval et al.; Kaeser, Aslanian et al. 2008), as against BRM

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and ARID1A. Given that the BRG1 containing complex is known to activate genes that participate in cell cycle arrest downstream of TGFβ signaling (Xi, He et al. 2008) and to activate expression of tumor suppressors such as p16, the results presented here suggesting an anti-oncogenic role for ARID1B assume significance. Together, the results of epigenetic studies and functional studies could pave the way towards identification of tumor suppressor function of ARID1B in PaCa.

5.3 Chromosomal deletion at 18q23 harbor PARD6G, a possible TSG in PaCa

Among several chromosomal abnormalities, deletion at 18q23 is a frequent event identified in several cancers including PaCa (Jones, Raval et al. 1997; Blons, Laccourreye et al. 2002; Gebhart, Thoma et al. 2002; Knosel, Petersen et al. 2002; Heidenblad, Schoenmakers et al. 2004; Bashyam, Bair et al. 2005; Mosse, Greshock et al. 2005). I therefore endeavored to study the chromosomal deletion located at 18q23 in the current study. First, CghFused Lasso was performed on aCGH data of PaCa cell line (Bashyam, Bair et al. 2005) and confirmed the deletion at the locus (Fig 4.3.1a). The locus harbors single annotated gene PARD6G as seen in the UCSC human genome browser (Fig 4.3.1a). Identification of possible TSGs through characterization of localized homozygous deletions has been frequently reported earlier. A classical TSG, DPC4 was identified in PaCa by characterizing a frequent deletion 18q21.1 (Hahn, Schutte et al. 1996). SMAD4 (also known as DPC4) was found to be deleted in several other cancers as well (Schutte, Hruban et al. 1996). TSGs can be inactivated in many ways, such as mutation in gene sequence and mutation in regulatory region of gene (Pfeifer and Besaratinia 2009). p53 is classical TSG, which is well known to be inactivated in human cancers including PaCa through mutation in DNA binding domain (Muller and Vousden 2013). PTEN, another well-studied TSG, has also been shown

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to be inactivated through mutation in several cancers including gastric carcinoma, oral squamous cell carcinoma (Li, Zhao et al. 2009; Wen, Wang et al. 2010). Classical TSGs such as *P16*, *p53* and *SMAD4* have been well documented to be inactivated through mutation in PaCa and its precursor lesions (Welsch, Kleeff et al. 2007). In addition to promoter hypermethylation, mutation in promoter region is one of the mechanisms for TSG inactivation. Mechanistically, mutation in promoter region can delete or add new binding elements for transcription factor and ultimately lead to deregulation of transcription. For example, a recent study identified a novel mutation in *TERT* gene promoter in familial and sporadic melanoma patients and in cell lines which created a new binding motif for transcription factor ETS/TCF and increased transcription of the gene (Horn, Figl et al. 2013). To ascertain the possible tumor suppressor function of *PARD6G* in PaCa, I carried out mutation screening in PaCa cell lines which harbor a single copy deletion as identified in CghFused Lasso analysis (Fig 4.3.1bB). Since one copy was probably inactivated through deletion, it was assumed that the other copy could possibly be inactivated through mutation. Mutation screening identified a novel six bp (GGGCGC) insertion in the putative promoter region of *PARD6G* in CFPAC-1 cell line (Fig. 4.3.2). *MnSOD* protein protects cells from oxidative stress and exhibit a reduced expression and enzymatic activity in several cancers (Dhar and St Clair 2012). Sequence analysis of *MnSOD* in several human cancer cell lines having reduced level of expression identified a novel mutation in proximal promoter region (Xu, Krishnan et al. 1999). Similarly, *PARD6G* exhibits reduced expression in CFPAC-1 cell line (Fig. 4.3.5.2A). This mutation implicates a possible mechanism of reduced expression in CFPAC-1. Mutation and CpG island hyper-methylation often contribute together for TSG inactivation in cancer. For example, *ARID1A* was found to be frequently mutated in breast cancer tissues and cell lines (Cornen, Adelaide et al. 2012). Interestingly, a recent study identified *ARID1A* promoter hyper-methylation in several breast cancer tissue samples.
exhibiting low level of *ARID1A* transcript (Zhang, Sun et al. 2013). To understand underlying mechanism for *PARD6G* down regulation, putative promoter, exon 1 and intron 1 regions were analyzed for prediction of CpG island and could identify a strong CpG island (Fig I.1). BGS analysis of intron CpG island revealed no significant hyper-methylation in intron 1 (Fig I.2). However, promoter BGS analysis may possibly confirm the hyper-methylation in the PaCa cell lines as a plausible mechanism for reduced expression of *PARD6G*.

Cancer cells regulate expression of several oncogenes and TSGs at the transcription level during tumor progression (Trzyna, Duleba et al. 2012). This regulation usually occurs in regulatory region of genes including promoter and repressor elements usually found near TSP and enhancer elements usually found further away. Promoter region plays a major role in transcription. This is regulatory region of the gene where RNA Polymerase II and essential transcription factors bind and transcription of gene takes place. In addition to promoter region, transcription activators and repressors bind in binding elements of regulatory region (Trzyna, Duleba et al. 2012). In cancer cells, transcription repression of TSG is a common mode of TSG inactivation through binding of transcription repressors. For example, T-cadherin is a TSG in melanoma and has been shown to be transcriptionally inactivated through binding of repressor *BRN2* (also known as *POU3F2* and *N-OCT-3*) in the proximal promoter region (Ellmann, Joshi et al. 2012). Well studied TSG, *p53* has been shown to be regulated at transcription level through binding of CCCTC-binding factor (CTCF). CTCF binds to *p53* promoter and inhibits binding of transcription repressor (Soto-Reyes and Recillas-Targa 2010). *p53* has been shown to bind to its response elements in regulatory region of several oncogenes (e.g. *c-Myc, STMN1, VEGF* and *CDC25C*), thereby transcriptionally repressing these oncogenes in several carcinoma (Rinn and Huarte 2011). Another important TSG is *BRCA1*, a major player in breast cancer progression. *BRCA1* has been shown to be repressed transcriptionally through the binding of *MTA1* (a component of

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NuRD nucleosome remodeling complex) in estrogen response element (Molli, Singh et al. 2008). PARD6G was identified in year 2001 as human homologue of C.elegans par-6 gene and found to be a part of polarity complex (Noda, Takeya et al. 2001) and its deletion (18q23 locus) in human cancers is well documented as described above in section 5.2. However, no information is available regarding mechanism of its regulation at transcription level. The determination of PARD6G TSP performed in this study (Fig. 4.3.3a, b and c) is the first endeavour to understand transcriptional regulation of PARD6G and as expected my in silico analysis of PARD6G gene sequence predicted TSP in up upstream of exon 1 and further experimental determination of TSP through primer extension was found to be in close proximity of predicted TSP (Fig. 4.3.4.1 A and B). Investigation of its regulatory elements in proximity of predicted promoter region through Luciferase reporter assay revealed a strong region of transcription repression and a region of transcription activation in PaCa cell line MiaPaCa2 (Fig. 4.3.5.2). Further, in silico analysis in TESS (Transcription element search software) identified many transcription activator and repressor elements in the region of transcription activation and repression (Fig. 4.3.5.3a,b and Tables 4.3, 4.4). However, in vitro analysis of regions of transcription activation and repression through DNA-protein binding assays is to be performed for understanding the mode of repression and activation. Results of my promoter analysis may be the first step towards understanding transcription regulation of PARD6G. Further analysis of promoter of PARD6G will provide a better understanding of transcription regulatory mechanism in PaCa.