CHAPTER-5

Discussion
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Cancer, a complex disease, involves genetic and epigenetic alterations. The importance of genetic alterations in the form of SNPs, mutations and chromosomal aberrations as well as its causative factors is now well known. However, recent findings have suggested that epigenetic alterations of gene function may also be central to the pathogenesis of this disease. Aberrant methylation in the form of loss (hypomethylation) or gain (hypermethylation) is commonly observed in cancer (Wajed, Laird et al. 2001). It is a known fact that genome wide loss of methylation takes place in cancer cells as opposed to its normal counterpart leading to activation of oncogenes, proto-oncogenes, and transposable elements; it’s over expression and chromosomal instability thereby acting as a route for carcinogenesis. In contrast to this the gain of methylation (hypermethylation) occurs in the CpG islands of promoter regions, impairs gene transcription leading to the silencing of tumor suppressor genes. Aberrant methylation of CpG island is a hallmark of cancer and may occur at an early stage in cervical carcinogenesis (Narayan, Arias-Pulido et al. 2003; Henken, Wilting et al. 2007; Yang, Nijhuis et al. 2010). The promoter is the site responsible for regulation of gene transcription and houses several regulatory DNA motifs including transcription factor binding site and in many cases CpG islands. Characterizing the promoter sequences will help to understand the underlined mechanism of gene expression. Methylation of CpG dinucleotides in the CpG islands which are located within the promoter sequences acts as molecular switch governing the gene expression. Hence it can be speculated that genes having CpG islands in the promoter region could be controlled by methylation. However, its impact on cervical carcinogenesis is not fully determined, and its potential as a diagnostic biomarker remains to be validated.

To acquire valuable information into the epigenetic switches that may promote and or contribute to the neoplastic events, we have analyzed the global DNA methylation profiles in normal and cervical cancer samples by RP-HPLC and genome wide candidate gene search by MS-AP-PCR, and also by using DMH based CpG island microarray platform followed by their validation in a panel of normal and tumor samples using bisulfite based techniques such as COBRA and BGS.
5.1. HPV infection and cervical cancer:

According to the current molecular epidemiological data, HPV is considered as the main risk factor responsible for cervical carcinogenesis. About 70% of cervical cancer is preventable by vaccinating women with HPV vaccines. Molecular epidemiological data suggest that geographical location contributes significantly to the HPV type distribution. Thus, before any HPV vaccine trial it is also very important to identify the most prevalent HPV in a given region so as to identify the efficacy of the existing HPV vaccine or to identify the need for a new one. As far as our knowledge is concerned not much work has been done in the area of HPV type distribution in the southern part of Karnataka and hence there is an urgent need to generate data on the most prevalent HPV types in southern part of Karnataka state.

In the present study we have genotyped the prevalence of HPV in non-malignant, pre-malignant and malignant cervical samples. Cervical cancer is the most common malignancy affecting Indian women. Strong evidence both at the epidemiological and the molecular level supports the necessary and causal involvement of a persistent infection with a high risk HPV in the pathogenesis of cervical cancer (zur Hausen 2002). Genital HPVs are sexually transmitted and it is estimated that about 80% of sexually active individuals encounter an HPV infection during their life, most of which pass unnoticed (Baseman and Koutsky 2005). The HPV prevalence depends on age and geographic area and is the highest (~20%) among women between 16 and 25 years of age. This number declines markedly (to about 5%) in women who are 40 years or older (Burchell, Winer et al. 2006). The highest prevalence rates have been detected in Africa and America, and the lowest rates in Europe and Asia (Burchell, Winer et al. 2006). HPV16 is the predominant genital type in cervical cancers causing ~55% of all tumors worldwide, followed by HPV18 (~16%), and HPV33 (~4%) (Clifford, Franceschi et al. 2006).

Eighty percent of all hrHPVs infections are transient and do not result in lesions. Of the remaining 20%, a majority develops into non-progressive CIN1 lesions that reflect a tolerant state of a productive infection and will regress spontaneously over time. A minority of the HPV infections persists and induces high-grade CIN lesions that are CIN2 and CIN3. It is estimated that only 5% of the CIN lesions, when left untreated, would result in cervical cancer, which
equals at maximum 1% of all hrHPVs infections. Thus, cervical cancer is a rare complication of hrHPVs infection. Infection with hrHPVs is thus necessary, but not sufficient to develop cervical cancer. Additional genetic and epigenetic events are necessary for the development of cervical cancer.

Current molecular epidemiological meta analysis studies have shown that HPV is the main causative agent for cervical cancer. To date several studies have reported the prevalence of HPV in general population and cervical cancer patients. The divergent HPV prevalence, type specific distribution and variation in the incidence of cervical cancer could be attributed to several factors such as different techniques used to detect the HPV, geographic location, socioeconomic status, and poor sexual hygiene. Cervical cancer is the most common malignancy effecting Indian women. HPV infection can be prevented by vaccinating the women with HPV vaccine. In this direction HPV vaccines will play important role in the prevention of cervical cancer in India. Every year, almost 74,000 women die due to cervical cancer in India, which is more than one fourth of the world deaths due to cervical cancer. Women in India have a 2.5% life time risk to get cervical cancer, which is double the risk as compared to the data worldwide (1.3%). The HPV vaccine will not only help to reduce the incidence and mortality of cervical cancer but will also reduce the cost burden for cervical cancer screening. Though HPV is the main causative agent for cervical cancer, its prevalence and distribution varies in different geographical regions of the world. In this direction identification of most prevalent HPV genotype to a particular region is very important before vaccination. Thus in this direction we aimed to identify and determine the distribution of major HPV types in malignant and non-malignant cases of cervical samples from women attending the OBG department of KMC, Manipal for cervical region screening with various complaints. Relatively little is known about the prevalence of HPV from Karnataka and the study will generate valuable information of most prevalent HPV genotype which in turn will help to understand the effectiveness of the HPV vaccines in the southern part of Karnataka.

Several meta analyses have identified the distribution of HPV types associated with cervical cancer (Bae, Lee et al. 2008; Koshiol, Lindsay et al. 2008; De Vuyst, Cliford et al. 2009; Sankaranarayanan, Nene et al. 2009), has classified 13 HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59) as carcinogenic (Castle 2009). In our study we have identified 10 HPV
types (HPV 16, 18, 31, 35, 42, 45, 6, 81, 33 and 58) and HPV16 and 18 were the most prevalent ones. HR-HPV prevalence was approximately 91% followed by 2% of IR-HPV infection and 7% of LR-HPV infection in contrast to a previous study by Sowjanya et al. (2005) wherein they have reported the detection of only HR-HPVs (87.8%) in a study undertaken in Andhra Pradesh state of India (Sowjanya, Jain et al. 2005). In a case control study reported from Chennai, India it was reported that the prevalence of HPV infection is as high as 99.4% in invasive cancer samples (Franceschi, Rajkumar et al. 2003). In our study we have reported 91% of prevalence of HR-HPVs. In another study reported by Travasso et al. (2008) the investigators used consensus PCR method followed by pyrosequencing for HPV genotyping and reported that the prevalence of HR-HPVs in 96.9% of cervical cancer samples (Travasso, Anand et al. 2008). They have also shown that high prevalence of HPV infection (76.19%) in non cancer samples and large proportion of cases (52.38%) were co infected with HPV16 and 18 (Travasso, Anand et al. 2008).

Several studies from India reported that HPV16 and 18 are the most prevalent HPV types found in India and the present vaccine against HPV16 and 18 holds great promise in preventing the women from HPV infection and subsequent development of cervical cancer. In a cross-sectional study reported by Bhatla et al. (2008) reported the prevalence of HPV infection among women with normal, low-grade cervical neoplasia (CIN 1), and high-grade CIN (>CIN2) were found to be 7.6%, 42.3%, and 87.5%, respectively (Bhatla, Dar et al. 2008). Recently it has been reported that HPV can even be detected from lymph node (Peedicayil, Sathish et al. 2009). Studies have also shown that HPV vaccine has the ability to reduce the cervical cancer incidence by 75% in India. The study reported the HPV prevalence in 87.1% of cases with HPV genotypes 16, 18, 31, 33, and 45 being the most common ones (Basu, Roychowdhury et al. 2009).

Several studies have reported the potential association of single nucleotide polymorphisms (SNPs) and HPV infection. The study has reported that CCND1 G1722C polymorphism may play a protective role against the development of human papillomavirus-associated cervical cancer among Indian women indicating that genetic makeup of individual along with other factors might play significant role in susceptibility of HPV infection (Thakur, Hussain et al. 2009). In a recent study observed 93.6%, 78.6%, and 10% to HPV infection in tumors, squamous intraepithelial lesions (SILs), and control (Singh, Datta et al. 2009). Apart from this, in a meta
study involving 131,746 healthy set of individuals it was observed that in low resource setting single round of HPV screening has the potential to reduce the incidence and mortality from cervical cancer (Sankaranarayanan, Nene et al. 2009). Tarik Gheit et al, 2009 reported the 93% prevalence of HPV in cervical cancer cases (Gheit, Vaccarella et al. 2009). Gupta et al, 2009 reported 16.6% prevalence of HPV in among cytologically normal women of reproductive age and HPV vaccine might play significant role in preventing and reducing the incidence and mentioned that mortality in cervical cancer (Gupta, Sodhani et al. 2009). Studies have also shown the prevalence of HPV in cytologically normal women of reproductive age group. Studies carried out in our country have reported the prevalence of HPV from 10.4% to 16.6% (Aggarwal, Gupta et al. 2006; Gupta, Sodhani et al. 2009). HPV prevalence (20.48%) in non malignant samples was higher when compared to other studies reported from India (Franceschi, Rajkumar et al. 2005; Sankaranarayanan, Nene et al. 2005) except for one study wherein they have reported 76.19% of HPV infection in non malignant samples (Travasso, Anand et al. 2008).

Several studies have reported that the prevalence of HPV infection decreases with age and HPV is uncommon in women above 35 years with normal cytology (Melkert, Hopman et al. 1993). Recent large meta analyses studies have reported an increase in the prevalence of HPV in pre- and post-menopausal state. Burchella et al (2006) reported increased HPV infection in younger women which decrease, with middle age and again show increase in older women (Burchell, Winer et al. 2006). Though our sample number is small, we have also observed similar result as that of Burchella et al wherein HPV infection was higher at early age and showed decrease in middle age and finally increase again at age 70 and above. The decrease and increase of HPV infection in one’s life could be attributed to the immunity and hormonal changes. With respect to parity in our study we observed women who are having parity between 2 to 3 (63.26%) had higher HPV infection as opposed to 0 to 1(45.45%) and more than 4(48.14%).

Women with more than 4 children showed decrease in infection with HR-HPV and multiple infections with increase in the number of births. Previous studies have reported conflicting results of parity as a risk factor for HPV infection: an increase, decrease and non-association between HPV infection and number of childbirths. Though a number of reports have showed association between numbers of childbirth as risk factor for HPV infection, data appear to be insufficient to give a conclusive result about the role of number of childbirth as risk factor for
HPV infection. The number of child birth may not be an independent risk factor for HPV infection but along with other factor such as immunity, smoking, alcohol and use of oral contraceptives might act as a risk factor for HPV infection. Thus in this direction more association studies needs to be undertaken to understand the role of parity in HPV infection. We did not observe any significant relationship between HPV infection and menopausal state (pre- and post-menopausal). Both single infection and multiple infections were found to be almost same between pre and post menopausal state.

Besides HPV16 and 18 other types of HPV infection accounted for about 13% of the cases. The HPV vaccines which are currently undergoing clinical trials predominantly targets HPV16 and 18. Cervarix (GSK) is reported to provide complete protection against HPV16, 18 associated infection while Gardasil (Merck) which is quadrivalent vaccine providing protection against HR-HPVs such as HPV16 and 18 and LR-HPVs such as HPV6 and 11. Though it can provide protection against the cervical cancer caused by HPV16 and 18, but may not benefit women infected with HR-HPVs other than HPV16 and 18. Since the cervical cancer incidence is very high in developing countries such as India the number of cervical cancer caused by HR-HPVs other than HPV16 and 18 are substantially high and is likely to be atleast 10,000-20,000 per year (Travasso, Anand et al. 2008) owing to high population density. Determining the rarer types of HPV and distribution will provide stress into alternative second generation vaccines against other HR-HPVs such HPV45, 31, 33, 35, 42, 52 and 58 to perform a major reduction in cervical cancer.

5.2. **Global hypomethylation is frequently observed in cervical cancer:**

The main aim of the study was to identify aberrant methylation in cervical cancer samples as opposed to normal samples. To estimate the global methylation content we have used RP-HPLC while for analysis of the genome wide hypermethylated CpG sites MS-AP-PCR and microarray were used.

Genome wide DNA methylation refers to the overall content of 5-methylcytosine in the genome. In the normal human somatic cells, depending on the type of tissue, between 70 – 90% of CpGs are methylated in DNA (Bird 1999). Because the CpGs are underrepresented in human genome,
this translates to 3 – 4% of all cytosine residues and 0.76% - 1.0% of all bases (Ehrlich, Gama-Sosa et al. 1982). Methylation of normal mammalian cell occurs primarily in the non-clustered CpGs in repetitive sequences (Gama-Sosa, Wang et al. 1983). Nearly 90% of all 5-methylcytosine lies within the transposons, including Alu, SINE, and LINE, which are comparatively rich in CpG dinucleotides and represent more than one-third of the human genome. Cytosine methylation within these CpG dinucleotides is thought to limit the ability of retrotransposons to be activated and transcribed and to participate in recombination. Unmethylated transposable elements permit genomic mutations, anomalous chromosomal recombinations eventuating in the unraveling of packed chromatin (Wilson, Power et al. 2007).

Global hypomethylation is frequently observed in many cancer cell types (Feinberg and Vogelstein 1983; Hoffmann and Schulz 2005; Richards, Zhang et al. 2009). While specific genes are hypermethylated in the genome of cancer cells, overall methylcytosine content is often decreased as a consequence of hypomethylation affecting many repetitive sequences. Hypomethylation is not only observed in repetitive sequences, retro-transposons but is also observed at a number of single-copy genes (Kim, Choi et al. 2006; Grunau, Brun et al. 2008; Vestergaard, Nexo et al. 2010). While global hypomethylation is highly prevalent across all cancer types, it often displays considerable specificity with regard to tumor type, tumor stage, and sequences affected. Based on the information available as of now several hypotheses have been proposed for hypomethylation and cancer. First, hypomethylation at a single-copy gene may occur as a 2-step process, in which selection for gene function follows upon random hypomethylation. In this fashion, hypomethylation facilitates the adaptation of cancer cells to the ever-changing tumor tissue microenvironment, particularly during metastasis. Second, the development of global hypomethylation is intimately linked to chromatin restructuring and nuclear disorganization in cancer cells, reflected in a large number of changes in histone-modifying enzymes and other chromatin regulators. Third, DNA hypomethylation may occur at least partly as a consequence of cell cycle deregulation disturbing the coordination between DNA replication and activity of DNA methyltransferases. Finally, because of their relation to tumor progression and metastasis, DNA hypomethylation markers may be particularly useful to classify cancer and predict their clinical course (Hoffmann and Schulz 2005). Decreased methylation of DNA can lead to loss of imprinting (LOI), chromosomal instability and this can drive cellular proliferation in cancer (Daskalos, Nikolaidis et al. 2009).
Our data suggest that global hypomethylation is characteristic of cervical cancer and might play a significant role in the development and progression of the same. Global hypomethylation was reported in many cancers previously (Moore, Pfeiffer et al. 2008; Richards, Zhang et al. 2009; Irahara, Nosho et al. 2010). Though the exact cause for global hypomethylation is not clear yet, it is speculated that several factors such as diet, viral infection, smoking, mutations in genes are involved in DNA methylation machinery causing global hypomethylation (Moore, Pfeiffer et al. 2008; Ogino, Kawasaki et al. 2008; Ogino, Nosho et al. 2008; Najjar Sadeghi, Zojaji et al. 2009; Richards, Zhang et al. 2009; Irahara, Nosho et al. 2010).

It is often not clear exactly which sequences are subject to global hypomethylation, nor the mechanisms that trigger this event. Several reports related nutritional status and vitamins in global DNA hypomethylation (Lee, Jacobs et al. 2009; Schernhammer, Giovannucci et al. 2009; Wang, Wang et al. 2010). Apart from this, tumor microenvironment like hypoxia and reactive oxygen species also play important role in global hypomethylation (Shahrzad, Bertrand et al. 2007; Tunc and Tremellen 2009). In colorectal cancer and melanoma, both in-vitro and in-vivo hypoxia lead to global hypomethylation (Shahrzad, Bertrand et al. 2007). It has also been reported that exposure of the primary fibroblast cell lines to hypoxic condition leads to reduction in 5mC and these fibroblasts undergo extensive demethylation when compared with metastatic cancer cell lines.

![Diagram of Factors and Events leading to hypomethylation and cervical cancer](image)

*Figure-72: Factors and Events leading to hypomethylation and cervical cancer*
Reactive oxygen species produce a broad spectrum of DNA damage, including single strand breaks, damage to the deoxyribose moiety and damage to the purine and pyrimidine bases (Calviello, Piccioni et al. 2006; Sedelnikova, Redon et al. 2010; Szaflik, Rusin et al. 2010). While thymine and cytosine constitute the bulk of pyrimidines in the DNA of most species, 5-methylcytosine (5-mC) may comprise up to 5% of all cytosine residues in mammalian genomic DNA. 5-mC residues in DNA are also 'hot spots' for mutation (Nomura, Tainaka et al. 2007; Cannistraro and Taylor 2009) as 5-Methylcytosine to thymine transition mutations occurs -10 times as frequently as cytosine to thymine transitions at other sites. 5-mC is subjected to various modifications in the presence of ROS such as hydroxyl radicals, super oxide anions and converted into mutagenic products such as 5-formylcytosine, thymine glycol (Kamiya, Tsuchiya et al. 2002). It has been shown that oxidative attack to methyl group of 5-methylcytosine, with the formation of 5-hydroxymethylcytosine, prevents the DNMT1 methylation of target cytosine. Hence reduced selectivity of cytosine by DNMT1 may be one of the reasons for decreased global methylation. Hence tumor microenvironment (especially ROS and hypoxic condition) along with other risk factors like viral infection and diet may play an important role determining the global methylation and subsequent inappropriate activation of gene involved in cancer development and progression.

Several studies have reported that the heritable transmission of cytosine methylation patterns following cell replication could be altered by endogenous DNA damage: oxidation tends to interfere with methylation, whereas 5-chlorocytosine and 5-bromocytosine can mimic 5-methylcytosine, resulting in fraudulent methylation of previously unmethylated sites. In a similar manner, oxidative damage tends to interfere with the binding of MBPs, whereas 5-chlorocytosine and 5-bromocytosine mimic 5-methylcytosine in facilitating binding. Although the precise mechanisms by which methylation patterns are established in cells are as yet unknown, endogenous DNA damage is likely to interfere with the faithful transmission of methylation patterns following cell replication and may account in part for epigenetic perturbations observed in human tumors (Valinluck and Sowers 2007).

Our study shows that global hypomethylation is a common phenomenon in cervical cancer. Based on our result and published literature we hypothesize that reactive oxygen species (ROS), tumor micro environment (Hypoxia), viral and other (dietary) factors together contribute greatly
to overall decrease in the 5mC content. ROS attacks on the methyl group of 5methylcytosine and converts into various modified mutagenic bases such as thymine glycol, 5-hydroxy methyl cytosine and other oxidized products. Thymine glycol, a mutagenic agent can cause GC-AT transition mutation resulting in the loss of methylation. Similarly, 5-hydroxy methyl cytosine, one more oxidation product of 5-methylcytosine, in presence of which DNMT1 cannot methylate the target cytosine again leading to the loss of methylated cytosine. Tumor microenvironment such as hypoxia induces local epigenetic alterations again can contribute to hypomethylation but the mechanism of hypoxia leading to hypomethylation is less studied. Similarly the DNA demethylase may also contribute to global hypomethylation and its role in global methylation needs to be examined. In HPV mediated carcinogenesis global hypomethylation was observed both in host genome as well as in viral genome (Ganem 2006; Crabbe 2010). Viral genome hypomethylation may activate the expression of viral onco-proteins namely E6 and E7 which in turn can degrade p53 protein resulting in, inefficient repair as well as may contribute immensely to the development and progression of the cancers. Apart from this viral genome also gets hypomethylated induced by ROS or hypoxia. Taken together, these factors suggest that they play important role in inducing global hypomethylation and induced carcinogenesis (Figure 72).

5.3. Genome wide methylation analysis by MS-AP-PCR:

In order to investigate the role of differential methylation in cervical cancer on a genome wide level and to identify the novel regions silenced in these tumors, we performed MS-AP-PCR approach that was previously reported to select frequently methylated CGI (Chango, Abdennebi-Najar et al. 2006; Ebert, Model et al. 2006; Estecio, Youssef et al. 2006; Tryndyak, Kovalchuk et al. 2006). By MS-AP-PCR we have isolated a total of 15 hypermethylated fragments in cervical cancer samples as opposed to normal cervical cells. Though some of the fragments are near or within the specific genes such as IKBKG, KLRG2, Myomesin-2, and NXN, no direct effect on expression regulation is expected as no promoter regions are involved. One of the fragments which were isolated as differentially methylated by MS-AP-PCR mapped to the promoter region of DOC2B gene. The presence of CpG island in the promoter region suggests a possible role of methylation in transcriptional regulation of the gene. The present study is the first to show that DOC2B gene promoter hypermethylation in cervical cancer samples leading to loss of mRNA
expression and demethylation using 5-Aza-2DC resulted in the re-expression of the gene. Apart from this we have also characterized the region which was used for methylation mapping by bisulfite genomic sequencing act as promoter and methylation and demethylation of this region is responsible for driving the DOC2B gene expression in in-vitro model systems by transient transfection assays.

Methylation of CpG sites within the regulatory regions of TSG is a common event in human cancers and is often associated with gene silencing. In the current study we have shown evidence for the first time that methylation is responsible for the silencing of DOC2B gene in cervical cancer cell lines. Double C2-like domains, beta (DOC2B) is a protein coding gene having at least two protein isoforms, namely alpha (DOC2A) and beta (DOC2B), which contain two C2-like domains and are encoded by different genes. DOC2B is expressed ubiquitously and is suggested to be involved in Ca (2+) dependent intracellular vesicle trafficking in various types of cells. The gene is located on chromosome 17 (17p13.3) and it codes for 412 amino acids. According to gene ontology analysis it is involved in calcium ion binding, calcium dependent phospholipids binding and transporter activity. DOC2 group of proteins were identified by screening the human brain cDNA library and is suggested to be involved in Ca2+ dependent vesicle trafficking neurotransmitter release (Orita, Sasaki et al. 1995; Sakaguchi, Orita et al. 1995). DOC2 beta also has two C2-like domains and is 61% identical to DOC2 alpha at the amino acid level. DOC2b gene interacts with syntaxin binding protein and Munc13 (Ke, Oh et al. 2007). The double C2 domain protein family (DOC2) is characterized by two calcium-binding domains (C2). Upon binding to calcium, the affinity of the protein to phospholipids is significantly increased, leading to translocation of the protein from the cytosol to the plasma membrane. These properties, and the binding domain of DOC2B to Munc13, suggested that DOC2B could play a role in augmentation and potentiation of synaptic release (Malkinson and Spira 2006). Recently it has been shown that that DOC2b is a positive SNARE regulator for GLUT4 vesicle fusion and mediates insulin-stimulated glucose transport in adipocytes as well as may be a regulator for delayed (second-phase) insulin secretion in MIN6 cells (Fukuda, Emoto et al. 2009).

Although it is known that DOC2B is ubiquitously expressed, its altered expression in cancer is not reported so far. Using MS-AP-PCR, MS-DMSO-PCR and BGS, we have identified the DNA methylation and the expression profile of DOC2B in cervical cancer cell lines and primary
tumors. In the present study we have identified promoter region of DOC2B gene as hypermethylated by MS-AP-PCR, we focused to examine (i) the methylation status of promoter region of DOC2B, which was identified as hypermethylated by MS-AP-PCR (ii) the possible role of DNA methylation on the functioning of DOC2B expression and (iii) finally to check whether if the DOC2B expression is silenced because of methylation whether its expression can be restored using demethylating agents. The methylation status of 30 cervical cancer cases, 15 normal samples and 3 cervical cancer cell lines were analyzed using MS-DMSO-PCR as described previously (Kholod, Boniver et al. 2007). The results of our MS-DMSO-PCR study demonstrate that the DOC2B promoter is more methylated in cell lines and tumor samples as opposed to normal samples. In order to map the methylated CpG sites BGS was used. The results of BGS showed that promoter region of DOC2B analyzed in the study are heavily methylated in cervical cancer cell lines SiHa, CaSki and HeLa leading to the complete loss of expression of DOC2B mRNA. Out of the three cell lines SiHa was less methylated (91.11%) while CaSki and HeLa were completely methylated. The partial methylation was more frequently observed in SiHa cells followed by CaSki while in HeLa partial methylation was not seen. There is a difference in the percentage of methylation at specific CpG sites in the cervical cancer cell lines and primary cervical carcinoma samples.

In cervical cancer cell lines the mRNA expression status was co-related with the methylation status of the cell lines after treatment with demethylating agent 5-Aza-2-DC. All the cell lines were heavily methylated and did not show the expression of DOC2B mRNA. Upon treatment with demethylating agent DOC2B mRNA expression was restored. SiHa cell lines which was least methylated and had higher number of partially methylated CpG showed better reactivation of mRNA, while HeLa cell line which was methylated at all the CpG analyzed showed least reactivation of DOC2B mRNA. All the cell lines showed different levels of DOC2B mRNA reactivation after demethylation. Our results show that DOC2B gene expression in cervical cancer cell lines is down regulated by transcriptional silencing due to epigenetic modification.

MS-DMSO-PCR and BGS have shown that the promoter region of DOC2B in primary cancer samples is hypermethylated as opposed to normal samples. In tumor, majority of the samples showed partial methylation with range of 0-86.66%. Since none of the normal samples showed hypermethylation, the partial methylation in primary tumors may be because of tissue
heterogeneity. Hypermethylation was not specific to any stage/grades of the disease as it is evident from the study that the hypermethylation is seen even in premalignant samples indicating that frequent hypermethylation of DOC2B may be an early event in cervical carcinogenesis and hence can be used as a marker in combination with other existing markers.

Infection with HPV is considered as the major cause for cervical cancer. A number of epigenetic alterations occur both in host as well as in viral genome during HPV induced carcinogenesis (Flanagan 2007). Recent experimental data suggests that both viral and host genes are targeted by the cellular epigenetic machinery leading to the aberrant activation and transcriptional deregulation and could be a potential mechanism leading to carcinogenesis (Uozaki and Fukayama 2008; Heather, Flower et al. 2009; Zheng, Zhang et al. 2009). At DNA level it interacts with DNA methyl transferases (DNMTs), methyl CpG binding proteins (MeCBPs) while at chromatin level it targets histone methyl transferases (HMT), histone acetyl transferases (HAT), histone deacetylase (HDACs) leading to alteration of gene expression which might play a significant role in tumorigenesis (Glaser, Staver et al. 2003). A significant association was observed between HPV infection and DOC2B methylation (P<0.007 by t-test). An independent association was observed between age and methylation indicating that the hypermethylation of DOC2B promoter might be due to the tumor specific nature of the cell and not due to the methylation modifying factors such as age which are reported to alter the methylation. The average methylation of promoter of DOC2B gene was higher in HPV positive samples when compared with HPV negative samples suggesting a strong association between HPV infection and hypermethylation of DOC2B gene. The bisulfite genomic sequencing results showed that DOC2B is methylated at more CpG sites /extent in HPV positive samples and this hypermethylation may be because of the over activation of DNMT brought about by HPV infection. Our result suggests that HPV infection might play significant role in the hypermethylation of DOC2B gene.

The BGS results show that promoter region of DOC2B gene is hypermethylated at some unique CpG sites in and around TSS which may play an important role in the expression of gene. Hypermethylation of CpG sites within and close to promoter region is involved in gene silencing. Hence it can be speculated that these tumor specific hypermethylation may play an
important role in the silencing of DOC2B gene. It has been shown previously that an altered concentration of Ca2+ in epithelial cells impairs the transport of desmosomal proteins to plasma membranes resulting in the disruption of desmosomal assembly and decreases the epidermal integrity. Since DOC2B is found to be hypermethylated in cervical cancer, it could be speculated that a defect in DOC2B might alter the cellular response to changes of calcium concentration and in turn may affect the transport of certain molecules involved in the differentiation of keratinocytes and may act as an alternative route for tumorigenesis. This gives us a hint that DOC2B may be involved in cancer growth and regulation within the body (Figure 41).

The domain mapping of DOC2B gene also showed to have BRCT domain. The BRCT domain is found predominantly in proteins involved in cell cycle checkpoint functions responsive to DNA damage (Bork, Hofmann et al. 1997), as found in the breast cancer DNA-repair protein BRCA1. The domain is approximately 100 amino acid tandem repeat, which appears to act as a phospho-protein binding domain (Yu, Chini et al. 2003). Functionally, BRCT containing proteins are involved in DNA repair, the DNA damage response and cell cycle regulation, most importantly by directing protein-protein interactions. BRCT domain showed to play important role in protein-protein interaction such as interaction with transcription factors such as E2F and p53. In this direction it can be speculated that loss of DOC2B expression by promoter hypermethylation might result in loss of DOC2B expression leading to the inappropriate activation of genes involved in DNA repair, DNA damage response and cell cycle regulation thereby leading to the accumulation of mutation which in turn might play significant role in tumorigenesis.

DOC2B gene is involved in Ca2+ transport; one can assume that alteration in its expression may lead to the accumulation of calcium ions. Calcium (Ca2+) is a universal intracellular second messenger that activates many cellular functions, such as gene expression, proliferation, differentiation, apoptosis and aging, cell adhesion, cell migration and others. An increase in cytosolic calcium concentration has been related to oncogene activation, wound repair as well as in the initiation and promotion of tumors. Further work on functional analysis is therefore needed to be carried out on DOC2B and its role in cancer.
5.4. Genome wide methylation analysis by microarray in normal and cervical cancer samples:

Microarray is a powerful technology currently being extensively used to identify the genome wide alterations such as methylation, CNV, expression, miRNA and SNPs in complex diseases such as cancer (Schena, Shalon et al. 1995; Hacia, Fan et al. 1999; Lim, Lau et al. 2005; Carter 2007; Kerkel, Spadola et al. 2008). Considering the importance of epigenetic alterations which is an early event in carcinogenesis, identification of set genes in each stage of tumor will help us to understand cancer and stage specific methylated biomarkers of diagnostic and prognostic significance. This in turn will help us to understand the signaling pathways governing the pathogenesis of cervical cancer. We have also used CpG island based microarray technique to identify the genome wide aberrant methylation in cervical cancer. Further, the results of the microarray experiments are validated by COBRA and BGS.

In this study, we presented the methylation profiles of clinical samples of cervical cancer using DMH combined with microarray and identified several CpG rich regions as differentially methylated between normal and cervical cancer sample. This sensitive genome wide screening approach has led to the identification of 139 hypermethylated CpG loci. Of the 139 hypermethylated genes, 120 genes were found to be annotated. Of the 120 annotated genes, 70 of them were found near the 5’UTR region and methylation in this region might play a significant role in the gene regulation. Further, 29 CGIs were mapped to the promoter region. In agreement with published data more than one half of the methylated CpG islands fall within the body of the genes or downstream of genes and functional role of these inter- and intra-genic CpG rich sequences remains to be elucidated (Illingworth, Kerr et al. 2008).

Most of the promoter region genes identified as hypermethylated in the study have either never reported to be methylated in cervical cancer or in other types of cancer. Apart from identifying the genes already reported to be hypermethylated in cancers we have also identified some novel genes whose promoter methylation might play significant role in cervical carcinogenesis. Our study not only identified a number of genes which are already reported to be hypermethylated in various cancer such as DAPK1, RAB6C, CDKN2A, ITGAV, SLC26A4 and TES but also a number of novel genes whose methylation status needs to be confirmed. Apart from the promoter regions, gene bodies, downstream regions and currently uncharacterized regions were also found...
to be hypermethylated. For this study we limit our analysis to promoter regions as these are well characterized for their effect on silencing gene expression and work needs to be done in this direction to identify the role of these non promoter sequences in cervical carcinogenesis.

By using 12K CpG island microarray platform we have identified several novel hypermethylated regions associated with known CNVs. Copy number variation (CNV) is a segment of DNA in which copy-number differences have been found by comparison of two or more genomes. The segment may range from one kilo base to several mega bases in size (Cook and Scherer 2008). Recent advances in microarray technology have lead to the development of microarray chips which can be used to identify the CNV. This has gained importance during last few years owing to its ability to alter function of the gene and thereby altering the gene expression. The first evidence that copy-number alterations can influence human phenotypes came from sporadic diseases, termed 'genomic disorders', caused by de novo structural alterations (Cook and Scherer 2008). The number of genomic disorders has grown, with several dozen reported to date (Lee, Carvalho et al. 2007). In addition to such sporadic diseases, inherited CNVs have been found to underlie Mendelian diseases in several families. Nonetheless, CNVs have been implicated in only a few percent of the 2,000 or more Mendelian diseases so far explained at molecular level (McCarroll and Altshuler 2007). The CNV is reported from several genes such CYP2D6, CD1d, GSTM1, TOP2A and GSK3beta (Lachman, Pedrosa et al. 2007; Nielsen, Ejlertsen et al. 2008; Huang, Chen et al. 2009; Yu and Shao 2009; Zhang, Song et al. 2010) and reported to play central role in different types of diseases including cancer. In our microarray study several regions identified as hypermethylated are often associated with CNVs. At present it is unclear on the functional role of CpG island associated CNVs. Hence further studies need to be undertaken to identify whether CpG islands associated with CNV are more prone for methylation and also the biological consequences of the hypermethylated regions associated with CNVs to uncover their role in cervical carcinogenesis.

Several studies reported have pointed out the usefulness of secreted proteins as biomarker for cancer (Pitteri, JeBailey et al. 2009; Rangiah, Tippornwong et al. 2009; Lai, Chou et al. 2010; Srisomsap, Sawangareetrakul et al. 2010). In this regards identifying a set of differentially expressed secreted proteins will help to identify and develop cancer biomarkers. In our study, we have bioinformatically identified 5 genes (secreted proteins) as hypermethylated. Hence further
studies need to be undertaken to unravel the secreted protein identified for its usefulness as biomarker and finally to develop a non invasive method for cervical cancer detection.

In order to address the functional relevance of the differential methylation of CpG island identified, gene ontology (GO) and pathway analysis were performed. To identify the pathways which might be altered due to the promoter methylation, the list of genes hypermethylated was mapped to different pathways using NCI-PID, Biocarta and KEGG pathway databases. The pathway analysis revealed that 16% of genes mapped to different biological pathways in KEGG. Pathways in cancer, metabolism, oxidative phosphorylation, pyrimidine metabolism, Wnt signaling and TGF beta are some of the pathways identified as altered in cervical cancer from our study. In conclusion, pathway mapping has uncovered not only the existing pathway but also the role of novel pathways in cervical carcinogenesis.

GO categories related to metabolic process, localization, cell communication and developmental process were the enriched categories in hypermethylated genes. Apart from this the study also identified a number of genes which are already associated with various diseases. When the genes were searched for their disease association it was found that large proportion of hypermethylated genes (31.34%) are indeed involved in a number of diseases and these genes might play a significant role cervical carcinogenesis. Interestingly majority of the genes identified were related to cancer (6/21; 29%) and diseases related to infection and immune functioning (4/21; 19%). Cervical cancer is the cancer caused by the infection of HPV. Several published studies have shown the importance of immune dis-functioning and subsequently leading to cervical cancer (Cheriyan, Krishna et al. 2009; Loddenkemper, Hoffmann et al. 2009; Patel and Chiplunkar 2009). HPV infection is more common in immune suppressed women and thus it can be speculated that in cervical cancer there is improper functioning of the genes related immune functioning leading to infection with HPV and ultimately leading to cervical carcinogenesis. Taken together, these results suggest that DMH approach not only validates previously known methylation regulated genes, but also identifies new candidate genes. Further these methylation regulated genes participate in major signaling pathways involved in cervical cancer and further work needs to be done to identify the role of these altered genes in cervical carcinogenesis.
From our study it is evident that aberrant methylation is not confined to single target gene in cervical cancer but rather can occur concurrently across many different genes spanning different chromosomes. We have identified several novel targets and have never been linked to epigenetic error in cervical carcinogenesis. Based on the results of the microarray, many genes identified by this methylation profiling approach are involved in pathways such as signaling events mediated through HDAC, E2F transcription factor network, INF gamma pathway, p53 pathway, E2F transcription network and these pathways are reported to be altered in various cancers including cervical cancer. Similarly PPP2CA and POT1 the genes which are involved in p53, pRB and regulation of telomerase were also found to be hypermethylated suggesting that a complex network of genes, pathways and signaling events together play significant role in the initiation and progression of cervical carcinogenesis.

By CGI microarray we have identified 29 promoter sequences as hypermethylated out of which DAPK1, RAB6C and ZNF471 promoter sequences are being validated in a panel of normal, malignant and cervical cancer cell line samples using BGS and COBRA. Apart from this DOC2B gene promoter is also mapped for methylation by BGS which was identified as hypermethylated by MS-AP-PCR. This is perhaps the first report of aberrant DNA methylation of DOC2B, RAB6C and ZNF471 in cervical cancer and it is the first one to show the promoter hypermethylation of DOC2B and ZNF471 in any disease. Apart from this we also showed that DOC2B and ZNF471 promoters are hypermethylated leading to transcriptional silencing of the gene which can be reactivated using treatment with demethylating agents such as 5-Aza-2DC.

### 5.4.1. Frequent hypermethylation of DAPK1 promoter in cervical cancer

![Figure-73: Schematic diagram of DAP-kinase protein structure](http://atlasgeneticsoncology.org/Genes/DAPKIID417ch9q21.html)
Death-associated protein kinase 1 (DAPK1) is a positive mediator of gamma-interferon induced programmed cell death. DAPK1 encodes a structurally unique 160-kD calmodulin-dependent serine-threonine kinase that carries 8 ankyrin repeats and 2 putative P-loop consensus sites (Figure 73). It is a tumor suppressor candidate located in 9q34.1

The 160 kDa actin microfilament-associated Ca\(^{2+}\)/calmodulin (CaM)-regulated Serine/Threonine kinase bears a multiple domain structure. The catalytic and the calmodulin regulatory domains determine substrate specificity and regulation of kinase catalytic activity, respectively. The non-catalytic association domains, involved in sub cellular localization or interactions with other proteins, include the 8 ankyrin repeats, two nucleotide-binding P-loops, a cytoskeleton-binding region, and a death domain. Phosphorylation by RSK at Ser289 triggers a suppression of DAPK pro apoptotic function. ERK phosphorylates DAPK at Ser735, which stimulates DAPK-mediated apoptosis. The DAPK1 promoter is activated by TGF-beta. This requires several of the SMAD proteins (SMAD2, SMAD3, and SMAD4). Overexpression of DAPK1 leads to cell death by apoptosis in the absence of TGF-beta, whereas inhibition of the kinase protects cells from apoptosis induced by TGF-beta (Raveh, Droguett et al. 2001), concluded that DAPK1 may play a role in the elimination of pre-malignant cells from development by serving as an early apoptosis checkpoint by suppressing oncogenic transformation.

The DAPK family has been linked to several cell death-related signaling pathways, and functions other than cell death have also been proposed (Bialik and Kimchi 2006). Death-associated protein kinase (DAPK) is the founding member of a newly classified family of Ser/Thr kinases, whose members not only possess significant homology in their catalytic domains, but also share cell death-associated functions (Bialik and Kimchi 2006). The realization that DAPK is a tumor suppressor gene, whose expression is lost in multiple tumor types, has spurred a flurry of interest in the kinase family and produced an impressive body of literature concerning its function, regulation, and connection to disease (Bialik and Kimchi 2006). DAP-kinase is a positive mediator of apoptosis induced by certain cytokines and oncogenes (Jang, Chen et al. 2002). Notably, high frequencies of DAP-kinase methylation have been found in B cell lymphomas and myeloma, where loss of control of c-Myc induced hyperproliferation from inactivated DAP-kinase may possibly play an important role in the pathogenesis of these B cell neoplasm.
DAPK1 is one of the most significant positive mediators of the programmed cell death induced by gamma-interferon found to be hypermethylated lung cancer, pituitary tumors, epilepsy, lymphoma, colon cancer, cervical cancer and other epithelial cancers (Schmezer and Plass 2008; Buyru, Altinisik et al. 2009; Qian, Wang et al. 2009; Qian, Yao et al. 2009; Wentzensen, Sherman et al. 2009). Recent studies have also shown that DAPK1 methylation is not a common phenomenon in cancer in general (Ciappetta, D'Urso et al. 2009; Lin, Geng et al. 2009; Borinstein, Conerly et al. 2010). DAPK1 hypermethylation in cervical cancer was reported by several studies (Feng, Balasubramanian et al. 2005; Henken, Wilting et al. 2007; Wentzensen, Sherman et al. 2009). Expression silencing through CpG island methylation of DAP-kinase has been frequently found in connection with adverse survival, as cells lacking DAP-kinase appear to be more invasive and more metastatic in lung cancer. Utility of DAPK1 promoter hypermethylation with increasing neoplasia along with other genes is also on record (Feng, Balasubramanian et al. 2005).

Figure 74: DAPK1 and its role in apoptosis

As per our study, DAPK1 was found to be hypermethylated in cervical cancer samples. The validation of DAPK1 promoter methylation in series of normal, premalignant and cancer samples revealed the hypermethylation in both pre-malignant and malignant samples. Similar observation was reported by Feng et al (2005) showing the hypermethylation of the four genes (CDH13, DAPK1, RARB, and TWIST1), the frequency of hypermethylation increased statistically significantly with increasing severity of neoplasia present in the cervical biopsy (Feng, Balasubramanian et al. 2005). The frequency of DAPK1 methylation in invasive cervical carcinoma was reported to be in the range of 45-100% (Dong, Kim et al. 2001; Narayan, Arias-Pulido et al. 2003). Since hypermethylation of promoter sequences are involved in silencing of
gene expression, these tumor specific hypermethylated CpG sites might play a significant role in the down regulation of DAPK1 and one can speculate that the site specific hypermethylation may play a significant role in the DAPK1 gene expression leading to the down regulation of the pathways, such as B-cell receptor, Notch signaling, TGF beta receptor and TNF alpha mediated pathway, which in turn leads to the escape of apoptotic signaling and help in malignancy. When the gene was mapped in silico it was found to interact directly with 14 and indirectly with 1481 different genes.

DAPK1 expression is reported to be suppressed in many cancers because of the promoter methylation. In our study also, we found it to be hypermethylated and hence the gene expression might be down regulated which might play a significant role in cervical carcinogenesis by escaping the carcinogenic signal. In our study on cervical cancer we have observed frequent hypermethylation of DAPK1 promoter in successive stages of cervical carcinogenesis, identified and mapped tumor specific methylated CpG sites. Thus taken together current experimental evidences suggest that DAPK1 promoter hypermethylation is a frequent and early event in many cancers and could be a potential candidate as marker for cancers. Thus methylation of DAPK1 needs to be validated in large number of clinical samples of various stages before using it as a marker.

5.4.2. Ras6C and Hypermethylation in cervical cancer:

Rab-GTPases are key regulators of membrane transport, and growing evidence indicates that their expression levels are altered in certain human malignancies, including cancer. Rab6C, a newly identified Rab6 subfamily member, has attracted recent attention because its reduced expression might confer a selective advantage to drug-resistant breast cancer cells (Young, Menetrey et al. 2010). Mammalian Rab (Ras-related proteins in brain) GTPases were first identified as evolutionarily conserved, essential regulators of membrane trafficking. The proteins are members of the wider Ras super family of GTPases. Over 70 human Rab and Rab like members of the Ras super family have been identified, and the functions of 36 Rab GTPases have been delineated. Rab GTPases are molecular switches, cycling between active and inactive states and serving as scaffolds to integrate both membrane trafficking and intracellular signaling
in a temporally and spatially sensitive manner. Despite the small sizes of Rab proteins (20-25 kDa), structural analysis reveal that they have multiple interaction surfaces through which they associate with regulatory molecules and downstream effectors to exert their functions. Rab proteins are increasingly found downstream of signaling cascades and can impact gene expression and growth control (Wennerberg, Rossman et al. 2005; Schwartz, Cao et al. 2007).

Given the importance of Rab GTPases in many cellular functions, it is not surprising that altered expression due to mutation or epigenetic alteration of Rab proteins and/or their effectors may underlie human diseases such as cancer (Rab25, Rab5 and Rab7), neuronal dysfunction (Rab1 and Rab7), retinal degeneration (Rab8), immune and pigmentation disorders (Rab27 and Rab38) and multidrug resistance (RAB6C) (Wennerberg, Rossman et al. 2005; Schwartz, Cao et al. 2007).

Rab6C or WTH3, a homologue of the Rab6 that belongs to Ras super family encodes a 254 amino acid polypeptide. Rab6c is capable of binding to GTP molecule and has been shown previously that WTH3 is down regulated in MDR cell lines and introducing it into those cell lines reversed the MDR phenotype to various anticancer drugs (Tian, Wang et al. 2008). Multidrug resistance (MDR) is the biggest problem and often complicates the effectiveness of chemotherapeutic agents in treating cancer patients by chemotherapy. Studies have shown that WTH3 is down regulated in MDR cell lines and by introducing the wild type gene reverted the MDR phenotype into normal phenotype by responding to various anti cancer agents (Shan, Yuan et al. 2002). WTH3 gene was discovered in MDR phenotype cell lines by MS-RDA technique (Shan, Yuan et al. 2002). Later its biological properties were characterized wherein it was shown that WTH3 gene products can bind to GTP by GTP gamma S binding assay and is mainly located on the membrane of the Golgi apparatus evenly spread in the cytosol which was identified by EGFP fluorescence markers. Tian et al (2005) showed that the down regulation of WTH3 in MDR type cell, when compared with non-MDR cell is because of its promoter methylation (Shan, Yuan et al. 2002).

A region (-481 to -305) in WTH3 promoter was hypermethylated in MCF7/AdrR but not MCF7/WT cells which was found to be responsible for reduced expression of WTH3 gene and in turn responsible for multidrug resistance (Tian, Wang et al. 2007; Tian, Wang et al. 2008). Thus
the WTH3 gene promoter was found to be differentially regulated in paired MDR vs non-MDR MCF cells due to their differential methylation and transcription factor modulation. Recently it has been shown that the promoter region of WTH3 gene had a p53 binding site and is directly regulated by p53 protein (Tian, Wang et al. 2007; Tian, Wang et al. 2008). Studies using semi-quantitative reverse transcriptase-polymerase chain reaction have shown that WTH3 was 15 and 4 times down regulated in MCF7/AdrR and MES-SA/Dx5, a human MDR uterine sarcoma cell line, as compared to their non-MDR parental cell lines (Shan, Yuan et al. 2002).

![Diagram](image)

**Figure-75: Rab6C and its role in multidrug resistance.** The cells whose promoter is unmethylated for RAB6C will have normal expression; will respond to various chemotherapeutic agents leading to apoptosis. While those cells whose promoter is hypermethylated for RAB6C will have reduced expression and subsequently may show poor response to various drugs, no or reduced apoptosis leading to proliferation of cancerous cells.

According to gene ontology analysis, RAB6C is involved in GTP binding, GTPase activity, nucleotide binding and protein binding. It is involved in the process of protein transport and response to drugs. Apart from this the interaction prediction has shown that RAB6C interacts directly and indirectly with several genes such as RAB6A, HRB, ABCB1, TBC1D9, FAM48A directly and RAB1A, RAB5A, TNF, JUN, PTPRC and ABCC1 indirectly. Our study is the first report showing the RAB6C promoter hypermethylation in cervical cancer. In cervical cancer we have identified that RAB6C promoter is hypermethylated and hence it can be speculated that this hypermethylation may lead to the down regulation of its expression leading to complication in treating the patients where RAB6C promoter is methylated. Thus promoter hypermethylation of RAB6C gene could be used as marker along with existing markers to identify and predict the
chemotherapeutic response in cervical cancer patients. Hence further work needs to be done before using it as a marker for identifying how a patient will respond to the various chemotherapeutic agents and also to establish alternative way of treating the patients who have methylated RAB6C promoter.

Thus Rab6c is an important gene whose expression is significant for determining the activity of chemotherapeutic agents and down regulation of RAB6C expression might make the cell resistant to various chemotherapeutic agents. Since promoter methylation plays an important role in the expression of Rab6C, it may act as marker for predicting the drug response in those patients where it is hypermethylated. Hence it can act as an important diagnostic and prognostic marker. Further it may act as an important tool for classifying patients as drug responders and non responders, which may help to design proper therapeutic regimes in those patients where it is hypermethylated (Figure 75). Thus further work needs to be done in the direction of Rab6c hypermethylation and drug response with respect to designing novel therapeutic strategy.

5.4.3. Zinc finger protein 471 is frequently hypermethylated in cervical cancer:

Zinc finger protein 471 (ZNF471) is a transcription factor, maps to 19q13.43. It is made up of 21059 bases having 5 exons coding for 626 amino acids consisting of 15 C2H2 type zinc fingers and a KRAB domain belongs to krueppel C2H2-type zinc-finger protein family, transcription factor may be involved in transcriptional regulation. Zinc finger (Znf) domains are relatively small protein motifs which contain multiple finger-like protrusions that make tandem contacts with their target molecule. They were first identified as a DNA-binding motif in transcription factor TFIIIA from Xenopus laevis; however, they are now recognized to bind to DNA, RNA, protein and/or lipid substrates. Their binding properties depend on the amino acid sequence of the finger domains and of the linker between fingers, as well as on the higher-order structures and the number of fingers. There are many super families of Znf motifs, varying in both sequence and structure. They display considerable versatility in binding modes, even between members of the same class (for example, some bind DNA, others to protein), suggesting that Znf motifs are stable scaffolds that have evolved specialized functions.
The Krueppel-associated box (KRAB) is a domain of around 75 amino acids that is found in the N-terminal part of about one third of eukaryotic Krueppel-type C2H2 zinc finger proteins (ZFPs). It is enriched in charged amino acids and can be divided into sub regions A and B, which are predicted to fold into two amphipathic α-helices. The KRAB A and B boxes can be separated by variable spacer segments and many KRAB proteins contain only the A box (Bellefroid, Poncelet et al. 1991). The KRAB domain functions as a transcriptional repressor when tethered to the template DNA by a DNA-binding domain. Gene silencing requires the binding of the KRAB domain to the RING-B box-coiled coil (RBCC) domain of the KAP-1/TIF1-β co-repressor. As KAP-1 binds to the heterochromatin proteins HP1, it has been proposed that the KRAB-ZFP-bound target gene could be silenced following recruitment to heterochromatin (Peng, Begg et al. 2000). Although the function of KRAB-ZFPs is largely unknown, they appear to play important roles during cell differentiation and development.

**Figure-76: Hypothesis showing possible role of Znf471 promoter hypermethylation and its subsequent role in diseases such as cancer.**

In our microarray experiment promoter region of ZNF471 was found as hypermethylated as opposed to normal samples. When its interaction was predicted, it was found to interact with Faxo43, Uba52, Ubb, Rps27a and Ubc. Several studies have reported transcription factor deregulations by promoter hypermethylation and subsequent role in carcinogenesis has been reported in renal cell carcinoma, colon and breast (Belanger, Tojcic et al. 2010; Cooper, Zou et
al. 2010; Qu, Yan et al. 2010). Presently no functional studies or the pathway have been reported for ZNF471 gene. ZNF471 interacts directly with Faxo43, Uba52, Ubb, Ubc and Rps27a and its promoter methylation may play a significant role in the regulation of its interacting partners. Interacting partner namely Ubiquitin-B (UBB), Ubiquitin-C (UBC), RPS27A and Ubiquitin–A52 (UbA52) are involved in the regulation of chromatin structure and stress response, as well as in the degradation of cellular proteins. In lower life forms, such as invertebrates, stress-response proteins similar to UBB have been associated with ageing. Although unproven, a role for UBB in ageing and/or age-related diseases is possible. Further the functional analysis of the interacting partners of ZNF471 revealed that most of them are involved in ubiquitin-proteosome pathway either directly or indirectly and hence the down regulation of ZNF471 might have significant impact in the transcription of the interacting partners. Thus impairment of the ubiquitin-proteosome system (UPS) may result in the failure to remove and degrade mis-folded proteins and consequently causing the accumulation of mis-folded proteins in the cell. The aberrant interactions between mis-folded proteins and normal intracellular proteins are thought to underlie the pathogenesis in many diseases (Figure 76). In the present study we have shown evidence that ZNF471 promoter is hypermethylated, leading to the down regulation of its expression which can be restored by treating the cells with demethylating agent in cervical cancer cell lines (SiHa and CaSki). Further, work needs to be done to identify the role of promoter methylation on molecular pathways controlled by ZNF471 which in turn will help us to understand the molecular mechanisms responsible for ZNF471 promoter methylation and their subsequent role in cervical carcinogenesis, if any.

5.4.4. Hypermethylation of CDKN2A and Cervical cancer:

Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4), also known as CDKN2A, is a tumor suppressor protein, which in humans is encoded by the CDKN2A gene (Nobori, Miura et al. 1994; Stone, Jiang et al. 1995). CDKN2A plays an important role in regulating the cell cycle, and mutations in CDKN2A, increase the risk of developing a variety of cancers, notably melanoma. The gene generates several transcript variants which differ in their first exons. At least three alternatively spliced variants encoding distinct proteins have been reported, two of which encode structurally related isoforms known to function as inhibitors of CDK4 kinase. The remaining transcript includes an alternate first exon located 20Kb upstream of
the remainder of the gene; this transcript contains an alternate open reading frame (ARF) that specifies a protein which is structurally unrelated to the products of the other variants. This ARF product functions as a stabilizer of the tumor suppressor protein p53 as it can interact with, and sequester, MDM1, a protein responsible for the degradation of p53. In spite of the structural and functional differences, the CDK inhibitor isoforms and the ARF product encoded by this gene, through the regulatory roles of CDK4 and p53 in cell cycle G1 progression, share a common functionality in cell cycle G1 control.

CDKN2A is capable of inducing cell cycle arrest in G1 and G2 phases. It acts as a tumor suppressor gene, binds to MDM2 and blocks its nucleocytoplasmic shuttling by sequestering it in the nucleolus. This inhibits the oncogenic action of MDM2 by blocking MDM2-induced degradation of p53 and enhancing p53-dependent transactivation and apoptosis. It also induces G2 arrest and apoptosis in a p53-independent manner by preventing the activation of cyclin B1/CDC2 complexes.

The alteration of CDKN2A expression either by mutation or by promoter methylation is reported in many cancers. The aberrant methylation of tumor suppressor gene CDKN2A promoters has been extensively investigated in cervical cancer (Terra, Murta et al. 2007; Nehls, Vinokurova et al. 2008; Kim, Choi et al. 2010). The promoter hypermethylation of CDKN2A and subsequent role in altered gene expression is a question of debate or is controversial. Studies have reported direct, inverse and no correlation between promoter hypermethylation of CDKN2A on its expression. Though CDKN2A is frequently methylated in cervical cancer, however, p16 is found strongly over expressed due to HP-oncogene mediated release of E2F from RB. Recently, it has been demonstrated that CDKN2A methylation does not affect protein expression in cervical cancer (Nehls, Vinokurova et al. 2008).

Studies have also demonstrated hypermethylation of the CDKN2A 5′ CpG island is not a frequent event in HR-HPV-positive cervical carcinomas and cannot be an effective marker of cancer cells with up-regulated expression of CDKN2A (Ivanova, Golovina et al. 2007). Study by Yuan et al (2005), suggested that inactivation of the CDKN2A gene is a frequent event and positively correlated with pathological grades in cervical cancer, and that methylation of the p16 gene appears to be an important event in carcinogenesis of cervical cancer (Yuan, Li et al.
The studies have also shown the usefulness of detection of hypermethylation CDKN2A even from plasma of cervical cancer patients suggesting its utility as diagnostic marker along with other genes such as DAPK1, MGMT, APC and HIC-1 (Dong, Kim et al. 2001; Yang, Liu et al. 2004). Thus taken together published results suggest that CDKN2A promoter hypermethylation is a frequent and early event in cervical carcinogenesis and can be useful as a biomarker for screening of cervical cancer if used in combination with other genes.

Though the introduction of PAP screening has greatly reduced the incidence of cervical cancer worldwide cases of cervical cancer continued to occur. However, the sensitivity of Pap smear testing is low and dependent on the expertise and different screening infrastructure. Infection with HPV is the primary and necessary cause for cervical cancer and studies have reported that identification of HPV is more sensitive than cytology (Cuzick, Szarewski et al. 2003) except that not as specific for the identification of women with CIN2. In the present study we have identified using HPV alone for screening SCC with a sensitivity and specificity of 82.75% and 77.41% respectively while for combined premalignant and SCC it was found to be 78.94% and 95% respectively. In this direction HPV testing and cytology screening (Pap test) together is expected to greatly enhance the sensitivity and specificity in detecting as well as diagnosing cervical cancer at early stage and hence it is recommended that further work needs to undertaken to elucidate the potential role of combined testing as a molecular tool for screening or predicating women who are at higher risk of developing cervical cancer.

Molecular screening is currently being used as a diagnostic and prognostic tool for identifying a number of diseases (Zidovec Lepej, Dusek et al. 2009; Boot 2010; Pingle, Rundell et al. 2010). Several studies have reported the usefulness of methylation markers as diagnostic and prognostic tool, classification, stratification and drug response. The use of DNA methylation as a molecular marker for cervical cancer has been reported by various investigators (Yang, Eijsink et al. 2009; Chaopatchayakul, Jearanaikoon et al. 2010; Cheng, Chen et al. 2010; Yang, Nijhuis et al. 2010). In the present study we did a genome wide search for differentially methylated regions in cervical cancer samples using microarray. Apart from genome wide search for differentially methylated regions, we also searched for whether multiple genes are simultaneously methylated in the same cancer cell. We analyzed the CpG rich regions of 4 genes identified as hypermethylated by our microarray experiment in a panel of normal, pre-malignant and
malignant samples by COBRA and BGS except for CDKN2A where in only normal, malignant and cervical cancer cell lines were used. By these techniques, we were able to address whether aberrant de novo methylation was targeted to specific CpG sites or was extensive across the CpG island promoter regions associated with tumor related genes.

From the present study it is evident that aberrant methylation is not confined to single target gene but rather can occur concurrently across many different genes spanning different chromosomes in an individual patient. All genes analyzed showed at least one hypermethylated gene promoters. The hypermethylation frequencies of DAPK1, DOC2B, ZNF471, RAB6C and CDKN2A in normal, premalignant, malignant and cervical cancer cell lines are shown in fig-80. Though the genes are hypermethylated in normal, pre malignant, malignant and cervical cancer cell lines, the frequency of hypermethylation was more pronounced in premalignant, malignant and cervical cancer cell lines. The analysis showed that in normal samples only few CpG sites are methylated and as the cells pass through successive stages of tumor progression more and more CpG sites get methylated. Out of the 5 genes analyzed, RAB6C was methylated only in malignant cervical samples while it was unmethylated in normal and premalignant samples. CDKN2A was partially methylated in all the normal samples analyzed while in malignant samples it was partial to complete methylation.

The methylation of CpG sites of DOC2B, CDKN2A, RAB6C, ZNF471 and DAPK1 was analyzed in normal, pre-malignant, malignant and cervical cancer cell lines (SiHa, CaSki and HeLa) by COBRA and BGS. DNA hypermethylation of CpG islands was detected premalignant, malignant and cell line samples, even in normal/non neoplastic cervical samples, probably because of the presence of precursor cells for cancer. CDKN2A, ZNF471, DOC2B and DAPK1 was methylated in normal samples while it was unmethylated for RAB6C. Though these genes are methylated in normal samples, only few sites were methylated when compared with premalignant, malignant and cervical cancer cell lines. In pre-malignant samples ZNF471, DOC2B and DAPK1 genes were hypermethylation while RAB6C was unmethylated and we did not sequence any pre-malignant samples for CDKN2A. In malignant samples all the genes analyzed showed heavy or extensive methylation of their CpG sites. Except DAPK1, all the cell
lines showed hypermethylation for the gene analyzed. DAPK1 was methylated only in SiHa while it was unmethylated in CaSKi and HeLa.

In our study we have identified the hypermethylation of all the five genes analyzed in malignant samples as opposed to normal samples. Samples were classified as unmethylated (0% methylation), low-(≥25% methylation), moderate- (25-50% methylation) and heavy methylation (≤50% methylation) based on the number of methylated CpG sites. The present study is the first reported demonstrating the hypermethylation of RAB6C, ZNF471 and DOC2B in cervical cancer.

We have detected the methylation of ZNF471 promoter region in normal, premalignant and malignant samples. The normal samples were found to be partially methylated (67%) while 33% of samples were found to be unmethylated. None of the normal samples showed low and heavy methylation. In premalignant samples low, moderate and heavy methylation was observed in 14.3%, 57.1% and 14.3% of samples respectively. In contrast to this, none of the malignant samples were unmethylated, and showed a methylation frequency of 8.3%, 8.3% and 83.3% of low- moderate and heavy methylation respectively. In contrast to normal and premalignant samples 83.3% of malignant samples showed heavy methylation while none of the normal samples showed heavy methylation.

The promoter region of DOC2B gene was found to be hyper methylated as opposed to normal samples. Most of the normal samples (66.7%) were found to be unmethylated and when methylated it showed low to moderate level of methylation with a frequency of 16.7% in each category. In contrast to this none of the premalignant and malignant samples were unmethylated. The 57.1% and 42.9% of pre-malignant samples showed low to moderate level of methylation while none of the premalignant sample showed heavy methylation. In contrast to normal and premalignant samples, low, moderate and heavy methylation was observed in 60%, 20% and 20% of malignant samples respectively. The heavy methylation of DOC2B promoter was observed only in full blown cancer samples. The present study is the first study reporting the RAB6C promoter hypermethylation in cervical cancer samples. All the normal and premalignant samples were found to be unmethylated while the 67% of the cancer samples were hypermethylated, indicating that the RAB6C hypermethylation might be late event in cervical carcinogenesis.
DAPK1 promoter hypermethylation is reported in a variety of cancer including cervical cancer. The DAPK1 promoter methylation was observed in normal, pre-malignant and malignant cervical cancer samples. The 87% of normal samples were methylated and were of low level types with moderate and heavy methylation being absent in normal samples. All the pre malignant samples analyzed were methylated with a frequency of 62.5%, 25% and 12.5% in low, moderate and heavy methylation category. In contrast to this all the tumor samples were methylated. The analysis showed that 35%, 20% and 45% of tumor samples showed low, moderate and heavy methylation. The CDKN2A promoter is frequently mutated, methylated and down regulated in a number of cancers. None of the samples analyzed were unmethylated. In contrast to other genes studied, CDKN2A showed partial to heavy methylation in normal and tumor samples analyzed. Hence samples were classified as unmethylated, partially methylated, partial-heavy and of heavy methylation. All the normal samples analyzed showed partial methylation while partial to heavy methylation was completely absent. In contrast to this in malignant samples we have observed partial, partial to heavy and heavy methylation in 30%, 40% and 30% of the samples analyzed. Majority of the tumor samples showed partial-heavy to heavy methylation.

Regulation of gene expression is a highly complex process which involves a large number of epigenetic modifications such as DNA methylation. Methylation and demethylation in the promoter region of genes is shown to play important role in gene expression. Epigenetic deregulation leading to altered gene expression during malignant transformation can alter cellular phenotype resulting in aberrant cellular proliferation and survival. DNA hypermethylation at the promoter region of key tumor suppressor genes showed to play important role in the initiation and progression of tumors. Apart from identifying the differentially methylated genes in primary cervical cancer, we have also identified the methylated regions by microarray approach in two cervical cancer cell lines namely, SiHa and CaSki. We have also identified those genes which can be demethylated upon treatment with demethylating agent. In order to define the epigenetic modification and epigenomic events after demethylation on a global level, we have used integrated microarray approach involving DNA methylation and gene expression. The dual screening approach was shown to be useful not only for identifying differentially methylated regions but also identifying novel therapeutic targets (Shi, Yan et al. 2002; Shi, Wei et al. 2003). To our knowledge it is the first report in which both
methylation and ECISTs are studied together in cervical cancer. This integrated microarray approach allowed for both, the identification of individual genes and a systematic analysis of the relationship among the DNA methylation machinery, promoter targets and downstream responses regulated by the DNA methylation.

It has been reported that by treatment with epigenetic modifying drugs it is possible to restore the expression of genes silenced by epigenetic events by reversal of promoter hypermethylation status which results in global and specific changes in gene expression (Yang et al, 2009). The study demonstrated that inhibition of DNA methylation has both primary and secondary effect. By dual screening approach we have demonstrated that the primary and secondary response on gene expression resulted from treatment with demethylating agents. Reactivation of genes silenced by CpG methylation would presumably involve a series of steps, including removal of transcriptional repressors and methylation dependent chromatin remodeling factors such as MBD/MECP complex that are recruited by MBD proteins. Epigenetic complexes have been shown to possess chromatin-remodeling activity, leading to altered chromatic structure thereby preventing the active gene transcription. It is assumed that disrupting these complexes would presumably diminish their activity and result in a more open, transcriptionally active chromatin configuration. A physical association between methylated DNAs and chromatin remodeling complex has been shown, and our observation that reactivation of methylation-silenced genes achieved by the demethylation treatment is suggestive of a functional interaction between the epigenetic modifications in cervical cancer. However, the functional relationship is because of a direct or indirect interaction between the molecular targets and this remains to be elucidated (Shi, Yan et al. 2002; Shi, Wei et al. 2003).

The omics studies such as transcriptomics, proteomics, and genomics have generated vast amount of data during the last decades. High throughput technologies such as microarray and mass spectrometry have generated large amount of data, the analysis and the interpretation of the omics data remain a challenge and requires effective bioinformatics approaches. The biological interpretation and hypothesis generation of the omics data is very important for further functional analysis. Several bioinformatic tools have been developed during last few years for analyzing and interpreting the large list of genes such as DAVID, NCI-pathway data base, KEGG, BABELOMICs, Ingenuity, GeneGo, VISANT, ClueGO, BINGO, HPRD, String and Panther for
functional, gene to gene interaction and pathway analysis of large scale data. In the present study, we have used an integrated bioinformatic approach for the gene expression and methylation microarray of cervical cancer cell lines. Demethylation resulted in the induction of biological process such as cellular component organization, primary metabolic process, developmental process and multicellular organismal process. The ClueGO and Agilent literature survey plugin of cytoscape, KEGG pathway and Gene Set analysis tool kit provide a gene to gene and protein to protein data integration for functional analysis. GO analysis, pathway, network and interaction analysis provided more biological insight and the pathways altered by methylation in cervical cancer.

The integrated analysis suggests that both aberrant DNA methylation and altered gene expression play a cumulative role in deregulation of gene networks in cervical cancer cell lines. Finally our methylation and expression microarray data from cervical cancer tissue biopsy and cell lines will provide a valuable reference for further analysis. We speculate that integrated approach of methylation and expression data generated from the same experimental system will provide new biological insight. Our study has demonstrated for the first time in cervical cancer that integrated analysis involving methylation and expression provides a more complete and comprehensive analysis of transcriptional programming. We envisage that integrated epigenomic and expression together with bioinformatic analysis such as gene to gene interaction, pathway analysis will identify the deregulated genes with more specific and sensitive manner and will enhance understanding of pathogenesis of the disease, which could be then used to design novel therapeutic strategies.