REVIEW OF LITERATURE

2.1 CHARACTERIZATION OF CYP26 FAMILY MEMBERS

2.1.1. Biochemistry of CYP26 Enzymes

The CYP26 family of enzymes was discovered in studies screening for proteins that contribute to RA dependent regeneration of damaged zebrafish fins (**White *et al.*, 1994; White *et al.*, 1996**). Although the sequence of the cloned cDNA from the zebrafish fins was less than 30% homologous with other P450 enzymes, the sequence contained a heme binding domain establishing the clone, P450RAI, as a CYP enzyme **(White *et al.*, 1996**). This CYP was believed to be a RA hydroxylase as it was inducible by RA and metabolized RA. P450RAI was later assigned as CYP26A1. After its initial discovery in Zebrafish, the *CYP26A1* gene was cloned and characterized in humans **(Ray *et al.*, 1997; White *et al.*, 1997),** as well as other species, including mouse **(Fujii *et al.*, 1997),** rat (**Wang *et al.*, 2002**), chicken (**Swindell *et al.*, 1999**), and cow **(Kruger et al., 2005**). Two other members of the family, CYP26B1 and CYP26C1 were later identified in humans (**Taimi *et al.*, 2004).** A fourth member of the CYP26 family has been published in the literature, CYP26D1 (**Zhao *et al.*, 2005; Gu *et al.*, 2005; Gu et *al.*, 2006),** however, it has only been identified in zebrafish and it appears identical with zebrafish CYP26C1 **(Hernandez *et al.*, 2007)**.

2.1.2 Biology of CYP26A1 Enzymes

Studies using knock-out animals have shown that both CYP26A1 and CYP26B1 are essential proteins. *Cyp26a1, Cyp26b1*, and *Cyp26c1* mice have all been produced using homologous recombination (**Abu-Abed *et al.*, 2001)**. Cyp26a1 and Cyp26c1 appear to have some functional redundancy as double knock-out of *Cyp26c1* and *Cyp26a1* had a more severe phenotype than *Cyp26a1* single knock-outs. Embryos of both knock-outs had limb and facial deformities. *Cyp26a1* mice had abnormalities similar to those observed in teratogenesis caused by RA excess. Interestingly, the lethal and phenotypic malformations observed in *Cyp26a1* mice could be rescued by heterozygous disruption of *Aldh1a2* (**Niederreither *et al.*, 2002**). *Cyp26a1* mice died during mid-gestation, whereas *Cyp26b1* mice died shortly after birth, which was attributed to respiratory failure (**Yashiro *et al.*, 2004**). *Cyp26a1* and

Cyp26b1 were lethal, whereas *Cyp26c1* mice were viable and did not show any alterations in embryonic development or phenotype (**Uehara *et al.*, 2007**) at birth. Using *in-situ* hybridization, CYP26 mRNA has been identified in murine embryos in a developmental stage and tissue specific manner. CYP26A1 gene expression has been detected in murine embryos as early as 6 days postcoitum (d.p.c.) with the posterior anterior gradient changing dramatically in a short period between 7.25 and 8.5 d.p.c. This expression pattern of CYP26A1 has been shown to create an uneven distribution of RA concentrations in the embryo, which then directs the development and patterning of the hindbrain, vertebrae, and tail bud **(Lutz *et al.*, 2009) .**

2.1.3 Cloning of the Individual Members of the CYP26A1 Family

Based on knowledge from the studies of White *et al*., used a differential display approach to identify genes that are responsive to RA, using zebrafish as a model organism. White *et al*., tested for genes expressed during regeneration of the amputated caudal fin of zebrafish, in the absence or presence of at-RA. By this approach an RA-responsive cDNA fragment was isolated, which was then used to clone P450RAI (for RA inducible) from a cDNA library from zebrafish embryo. After transfection of CYP26A1 cDNA into COS-1 cells, the expressed CYP26A1 protein catalyzed the conversion of at-RA to 4-hydroxy-RA, 4-oxo-RA, and 18-hydroxy-RA (**White *et al.*, 1996**). Similar to all Cytochrome P450 superfamily members, the predicted protein from this clone contains a conserved heme-binding domain motif located in the C-terminal part of the protein. This domain consists of a cysteine residue at the center, involved in the heme binding, surrounded by a series of conserved amino acid residues. However, the predicted Cytochrome P450 protein was found to have less than 40% amino acid identity to other known Cytochrome P450s, and therefore the gene was classified as a member of a new Cytochrome P450 gene family, designated CYP26 (**White *et al.*, 1997)**, with P450RAI becoming CYP26A1. A year later, the same authors reported the human cDNA ortholog of CYP26A1 (**White and Beckett-Jones, 1997**), and at the same time two independent groups cloned the mouse cDNA version of CYP26A1 (**Fujii *et al.*, 1997; Ray *et al.*, 1997**). CYP26A1 cDNA has now been reported for several other species including

chicken, xenopus, rat, and cow **(Fujii *et al.*, 1998; Swindell *et al.*, 1999; Nelson, 1999; Wang *et al.*, 2002; Kruger *et al.*, 2005)**.

2.1.4 Expression Patterns

In general, Cytochromes P450 comprise a superfamily of membrane-bound oxidative hemo protein enzymes, which are expressed in many tissues in vivo but most often are present at the highest level in mammalian liver. These enzymes have been found to be the principal route of metabolism for many hormones, drugs, and other xenobiotics. The patterns of expression for each of the three CYP26 genes differ both in the adult and in embryonic tissues and have much broader expression patterns than many cytochrome P450s. In the adult, CYP26A1 is mostly highly expressed in the liver, but less in brain and testis **(Ray *et al.*, 1997; White *et al.*, 1997).** This might be attributable to the variability among individuals in vitamin A intake, since CYP26A1 is highly regulated by vitamin A in the liver. CYP26A1 is also expressed in cell lines from several tissue origins. Human epidermal keratinocytes, on the other hand, express only low basal levels of CYP26A1 and do not exhibit induction by RA **(Popa *et al.*, 1999).** For CYP26A1, cell lines tend to fall into two groups. One group expresses the gene at a very low level but exhibits a high response to vitamin A or RA; these include HepG2 and MCF-7 cells (**Loudig *et al.*, 2000; Wang *et al.*, 2002; Loudig *et al.*, 2005; Zhang *et al.*, 2010).** The other group expresses the CYP26A1 gene at a higher basal level but is less regulated by vitamin A or RA; this includes HEK293T and SK-LC6 **(Zhang *et al.*, 2010).** In the adult human liver, comparative studies found the level of CYP26A1 expression to be highly variable among individuals **(Thatcher *et al.*, 2010).**

2.2 MOLECULAR AND PHYSIOLOGICAL FUNCTIONS OF CYP26A1

The at-RA is not only the specific substrate for CYP26A1 and CYP26B1 isozymes but also a potent inducer of these genes is particularly evident for CYP26A1 in liver (**Fujii *et al.*, 1997; Ray *et al.*, 1997; Yamamoto *et al.*, 2000; 2002; Wang *et al.*, 2002**) and for CYP26B1 in lung tissue (**Chapman *et al.*, 2003**). CYP26A1 mRNA increased progressively with age for each level of dietary vitamin A, which

also correlated with age-related increases in liver vitamin A content, either a vitamin A–adequate or vitamin A–supplemented diet. Thus, under steady state conditions, differences in the intake of vitamin A and the duration of exposure to vitamin A correlate well with CYP26A1 mRNA levels. The CYP26A1 gene is responsive to differences in dietary intake of vitamin A, the most dramatic response is observed after administration of at-RA and, again, especially in the liver (**Zolfaghari *et al.*, 2005**). The continuous presence of RA is necessary to maintain elevated expression of CYP26A1. The CYP26A1 gene also exhibits moderate regulation by retinoid treatment in other tissues including testis, lung, kidney, and small intestine (**Zhang *et al.*, 2010; Wu and Ross, 2010**). Similar to the response of CYP26A1 in the liver of intact animals, CYP26A1 mRNA also increases rapidly in hepatocyte cell lines, such as HepG2 cells, after treatment with at-RA or its analogs (**Tay *et al.*, 2010; Thatcher *et al.*, 2010; Ross *et al.*, 2011**). Cellular Retinoic Acid Bioavailability Determines Epithelial Integrity, the epithelial barrier is determined primarily by intercellular tight junctions (TJs). several types of RA, including atRA, promote the barrier function of epithelial TJs. Conversely, RA depletion in the cells by overexpressing CYP26s, cytochrome P450 enzymes specifically involved in the metabolic inactivation of RAs, induces an increase of permeability as measured by two differently sized tracer molecules, inulin and mannitol. This RA-mediated enhancement of barrier function is potentially associated with the increased expression of TJ-associated genes such as occludin, claudin-1, claudin-4, and zonula occludens-1.

2.2.1 Catalytic Function of the CYP26A1 Enzyme

The **cytochrome P450** superfamily of monooxygenases is a large and diverse group of enzymes that catalyze the oxidation of organic substances. The most common reaction catalyzed by cytochromes P450 is a monooxygenase reaction, e.g., insertion of one atom of oxygen into the aliphatic position of an organic substrate (RH) while the other oxygen atom is reduced to water:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

Cytochromes P450 (CYPs) belong to the superfamily of proteins containing a heme cofactor and, therefore, are hemoproteins. Like other cytochrome P450 family members, CYP26A1 proteins are endoplasmic reticulum (microsomal)

proteins. They are difficult to isolate and apparently require a membrane environment for activity. The functional activity of the products of RA metabolism has been a subject of some debate. Results from several studies showed that some of the oxidative products of at-RA metabolism such as 4-oxo-RA and 5,6 epoxy-RA may have some biological activity. These activities include the induction of differentiation and subsequent proliferation of growth-arrest. Good growth response shows in vitamin A–deficient rats (**John *et al.*, 1967**), epithelial differentiation (**McCormick *et al.*, 1978**), modulation of positional specification in early embryo in *Xenopus* (**Pijnappel *et al.*, 1993**), and spermatogonia of vitamin A–deficient mice testis (**Gaemers *et al.*, 1996**). Thus, studies of enzymatic activity of the CYP26A1 proteins have relied on assays in microsomal fractions prepared from cultured cells and tissue samples **(White *et al.*, 1996; 2000; Yamamoto *et al.*, 2000**), especially from liver, or supersome assemblies as a source of cDNA-expressed enzymes. However based on genetic evidence, at-RA—but not the oxidative derivatives of at-RA metabolism—is involved in embryonic development (**Niederreither *et al.*, 2007; Jayne *et al.*, 2011**). In general, similar oxidized products have been reported for microsomal extracts from liver of mice and rats treated in vivo with at-RA (**Ross *et al.*, 2011**) as compared to those observed in transfected cells. CYP26A1 exhibits very high catalytic activity toward at-RA but a much lower activity toward other retinoids, including 9-*cis*-RA, retinal, and retinol. Thus, the data currently are inconsistent, and no firm conclusion can be drawn regarding the biological activity of oxidized retinoids. Whether these metabolites are active or inactive could be context specific, according to where they are produced and available.

2.2.2 Function of Vitamin A (retinol) in Embryonic Development

During embryonic development, RA plays a pivotal role in pattern formation both in the early embryo and during the period of organogenesis. Nearly all of the cells of the embryo are capable of RA signaling, as isoforms of each of the retinoid nuclear receptors are expressed during embryonic development **(Soprano and Soprano., 1995; Chambon., 1996).** Whereas CYP26A1 is expressed as early as embryonic day E 6.0 in extra embryonic and embryonic endoderm (**Fujii *et al.*, 1997**). Potential function has been studied in embryonic stem (ES) cells undergoing differentiation. In undifferentiated ES cells, removal of leukemia

inhibitory factor (LIF), which functions to prevent differentiation, resulted in increased CYP26A1 expression and increased conversion of all-trans retinol to 4-oxo-retinol; however, neither RA nor 4-oxo-RA were detected (**Lane *et al.*, 1999**). Vitamin A deficiency at the time of mating and during pregnancy results in a collection of defects and malformations in the fetus, referred to as vitamin A–deficiency syndrome (**Nadin and Murray, 1999; MacLean *et al.*, 2001; Stephenson, 2011).** The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures. The spatio-temporal distribution of embryonic RA results from regulated expression of RA-synthesizing retinaldehyde dehydrogenases and RA-metabolizing cytochrome P450s (CYP26). Excess RA administration or RA deficiency results in a complex spectrum of embryonic abnormalities **(Abu-Abed *et al.*, 2001; Abu-Abed *et al.*, 2001; Clagett-Dame *et al.*, 2002).** On the other hand, an excess intake of vitamin A, RA, or acidic retinoid analogs is highly teratogenic and induces abnormalities that are not very different from those that result from deficiency, including defects in craniofacial, central nervous, and cardiovascular systems (**Clagett-Dame and De Luca., 2002; See *et al.*, 2008)**. Tissue RA concentrations are regulated both by enzymes that generate RA—retinol and retinal dehydrogenases—and by enzymes that metabolize RA to less active metabolites, including all members of the CYP26 family **(Duester, 2008; Niederreither and Doll´e, 2008**). Because RA molecules can easily diffuse across distances or be transported by proteins such as albumin and are rapidly taken up by cells, it is presumed that the proper regulation of local concentrations of RA is essential for normal development (**Pennimpede *et al.*, 2010**). All members of the CYP26 family are expressed in human, rodent, and chicken embryos, but their special and temporal regulation is highly specific (**Pennimpede *et al.*, 2010**).

2.2.3 Function of Retinoic Acid in Germ Cell Development

During germ cell development, whether germ cells develop as oocytes or spermatogonia depends on the time at which the cells enter meiosis **(Edwin *et al.*, 1999)**. Whereas RA, which is known to stimulate meiosis (**Koubova *et al.*, 2006**), is produced in the mesonephros adjacent to the developing gonads of both sexes, as demonstrated by expression of an RA sensitive reporter gene, by 13.5 dpc only the male gonad expresses CYP26B1, apparently in sertoli cells, and the

relative amount of RA in the male gonad is reduced to 25% of that in the female gonad (**Bowles *et al*., 2007**). During murine embryonic development, all three CYP26 family isozymes are distinctly expressed in relatively non overlapping regions, which are particularly sensitive to the teratogenic effects of RA (**Duester, 2008; Niederreither and Doll´e, 2008**). In another study using AB1 ES cells, the disruption of both alleles of the CYP26A1 gene by homologous recombination resulted in an 11-fold higher concentration of intracellular RA in cells treated 48 hours earlier with RA together with reduced expression of RA-responsive genes involved in cell differentiation (**Langton and Gudas, 2008**).

2.2.4 Function of CYP26A1 in Metabolism of Retinoic Acid

The most common metabolite mentioned in the literature is 4-OH-RA, which can then be further oxidized to 4-oxo-RA and diOH-RA. Other identified metabolites include 18-OH-RA, 3-OH-RA and 5,6-epoxy-RA. There are reports that some RA metabolites, for example, 4-oxo-RA and 5,6-epoxy-RA have biological activity **(Roberts and DeLuca, 1967**; **John *et al*., 1967; McCormick *et al*., 1978**). At-RA is metabolized by several different enzymes to form multiple products such as ketones, epoxides, and hydroxylated products (**Napoli *et al*., 1978; Frolik *et al*., 1979)**. While the hydroxylated and subsequently oxidized metabolites are primarily formed through cytochrome P450 catalyzed mechanisms, the 5,6 epoxide appears to be generated through peroxyl radical-dependent mechanisms (**Pijnappel *et al*., 1993**). RA, its isomers, and its metabolites can also undergo phase II metabolism by UGT2B7 to form RA glucuronides. While at-RA, 5,6-epoxy-RA and 4-oxo-RA are exclusively carboxyl-linked to the glucuronide, 4-OH-RA can be glucuronidated at either the carboxyl or the hydroxyl function (**Samokyszyn *et al*., 2000; Tzimas and Nau, 2001**). **Ross and Zolfaghari, 2004** studied f*unctions and actions of Retinoids and Carotenoids in the r*egulation of hepatic retinol metabolism. Lecithin retinol acyltransferase (LRAT) , a microsomal enzyme present in liver and several other retinol-metabolizing tissues, esterifies retinol that is associated with a cellular retinol-binding protein, CRBP or CRBP-II. In this research it is shown that LRAT mRNA expression and enzyme activity are regulated in a tissue-specific manner. In vitamin A-deficient liver, both LRAT mRNA and activity are significantly down-regulated as well as rapidly induced after the administration of vitamin A or its principal hormonal metabolite,

retinoic acid (RA). The general consensus is that RA, not the metabolites, regulate development (**Ozpolat *et al*., 2005; MacLean *et al*., 2007; Jayne *et al*., 2009**).

2.3 RETINOIC ACID-METABOLIZING ENZYME CYP26A1 AND ITS ROLE IN HUMAN CANCER

CYP26A1 enzymes may play a similar but separate role in limiting the consequences of fluctuations in nutritional vitamin A **(DeLuca and Roberts, 1969).** The possibility that pathological conditions such as cancer might involve aberrant expression of CYP26A1 has recently emerged from several studies. Elevated CYP26A1 expression and RA catabolic activity have been detected in breast epithelial adenocarcinoma cells in culture **(Van Heusden *et al*., 1998).** Retinoic Acid hydroxylase (CYP26) is a key enzyme in Neuronal Differentiation of Embryonal Carcinoma cells (**Sonneveld *et al*., 1999**). Results indicate that the effects on growth inhibition and RARb transactivation of P19 Embryonal Carcinoma cells are mediated directly by RA, while the onset of neuronal differentiation and the subsequent expression of neuronal markers is mediated by *h*CYP26 via the conversion of RA to its hydroxylated products. The cells derived from squamous cell carcinoma from head and neck cancers (**Klaassen *et al*., 2001**) and leukemic cells are derived from patients with acute promyelocytic leukemia (APL) **(Ozpolat *et al*., 2002),** however, the relevance of elevated CYP26A1 activity in tumor cells remains to be fully clarified. The regulation of programmed cell death (also known as apoptosis) is a process critical for both normal embryonic development and turnover of healthy tissue in the adult. In a number of disease states, however, the pivotal balance between proapoptotic and cell survival signals is disrupted, leading to loss of healthy cells as in neurodegenerative disorders or failure to eliminate genetically damaged cells, leading to cancer (**Evan and Vousden, 2001; Green and Evan, 2002)**. The mechanisms controlling cellular self-destruction programs are tightly controlled, and many physiological growth control signals that govern cell proliferation and tissue homeostasis are linked to apoptosis (**Gozani *et al*., 2002; Green and Evan, 2002)**. It has been previously demonstrated that expression of the RA-metabolizing cytochrome P450s CYP26A1, B1, and C1 protects cells and tissues from exposure to RA (generated by retinaldehyde dehydrogenase enzymes) during embryogenesis by restricting RA access to transcriptional

machinery by converting RA into rapidly excreted oxoderivatives (4-OH RA, 4-oxo RA, 18-OH RA) (**Tahayato *et al.*, 2003; Taimi *et al.*, 2004)**. **Baudet *et al.*, 2007** analyzed the pharmacogenomic of acute promyelocytic leukemia cells and highlights CYP26 cytochrome metabolism in differential all-*trans* retinoic acid sensitivity. Disease relapse sometimes occurs after acute promyelocytic leukemia (APL) therapy with all-trans retinoic acid (ATRA). To identify biologic networks involved in resistance, they conducted pharmacogenomic studies in APL blasts displaying distinct ATRA sensitivities. In opposition, only high-sensitive blasts expressed the *CYP26A1* gene, encoding the p450 cytochrome which is known to be involved in retinoic acid catabolism. In NB4 cells, ATRA treatment activates a novel signaling pathway, whereby interleukin-8 stimulates the expression of the homeobox transcription factor HOXA10v2, an effective enhancer of *CYP26A1* transcription. These data were corroborated in primary APL cells, as maturation levels correlated with *CYP26A1* expression. **Hong *et al*. (2008)** analysed the novel role for the retinoic acid-catabolizing enzyme CYP26A1 in Barrett's associated adenocarcinoma. Vitamin A deficiency is associated with carcinogenesis, and upregulation of CYP26A1, a major retinoic acid (RA)-catabolizing enzyme, has recently been shown in cancer. Previously he demonstrated alterations of RA biosynthesis in Barrett's oesophagus, the precursor lesion to oesophageal adenocarcinoma. Retinoic acid response element reporter cells were used to determine RA levels in non-dysplastic and dysplastic Barrett's cell lines and endoscopic biopsies. CYP26A1 was stably overexpressed in GihTERT cells, which were evaluated for gene-expression changes, cellular proliferation and invasion with or without the CYP inhibitor ketaconazole. RA levels decreased progressively with the degree of dysplasia ($P<0.05$) and were inversely correlated with CYP26A1 gene levels and activity ($P<0.01$). CYP26A1 expression was increased synergistically by RA and lithocholic acid ($P<0.05$). Overexpression of CYP26A1 led to induction of c-Myc, epidermal growth factor receptor and matrix metalloproteinase 3 as well as downregulation of tissue inhibitor metalloproteinase 1 and 3. Overexpression of CYP26A1 causes intracellular RA depletion and drives the cell into a highly proliferative and invasive state with induction of other known oncogenes. **Oasani and Lee, (2011)** analyzed the enhanced expression of retinoic acid-metabolizing enzyme CYP26A1 in sunlight-damaged human skin. Vitamin A deficiency (VAD) is associated with increased

susceptibility to carcinogenesis. CYP26A1, the gene encoding a cytochrome P450 enzyme specifically involved in metabolic inactivation of retinoic acid (RA), the most active vitamin A derivative, has been shown to result in a state of functional VAD of the cell. It has been recently, demonstrated that CYP26A1 efficiently promotes cell survival properties and eventually contributes to the carcinogenic process, implying roles as an oncogene. To clarify the possible association between VAD caused by CYP26A1 expression and the development of human epithelial neoplasia, and examine whether enhanced expression of CYP26A1 might be observed in various lesions of human skin. Expression of constitutive CYP26A1 in skin malignancies, such as squamous cell carcinomas, on the basis of observation suggests an involvement of enhanced CYP26A1 expression causing a functional VAD state in skin that can potentially lead to neoplastic transformation of keratinocytes in an early phase during skin carcinogenesis.

2.4 MUTATIONS OF CYP26A1 GENE IN HUMANS

Cytochrome P450 26A1 is a protein that is encoded by CYP26A1 gene in humans. This gene encodes a member of the Cytochrome P450 superfamily of enzymes. It has been mapped at chromosome 10q23-q24. It has 7 exons and two alternatively spliced transcript variants, which encode the distinct isoforms. Transcript variant 1, mRNA (refseq NM-000783.3) is having 2245 base pairs, and transcript variant 2, mRNA (refseq NM_057157.2) is having 2119 base pairs. One mutation has also been reported in above gene which is responsible for altered enzyme activity. There are total 3 mutations, reported in HGMD (Human Gene Mutation Database) **(Table 2.1)**. Micro deletions (20 bp or less) are presented in terms of the deleted bases in the case of altered enzyme activity phenotype, 10 bp DNA sequence flanking both sides of the lesion and the number codon is preceded at 413 position. Small indel (20 bp or less) are presented in terms of the deleted/inserted base **ga** in case of reduced mRNA efficiency phenotype and the number codon is preceded at 288 position. Splice mutations with consequences for mRNA splicing are presented in the form of reduced mRNA splicing efficiency. There is currently 1 mutation available in each above category of variants. The mutations in present gene is responsible for various kind of diseases in humans. The first mutational screening of CYP26A1 gene was reported by De macro *et al.* (2006) in patients with caudal

regression syndrome. In this enzyme CYP26A1 plays an important role in protecting tail bud tissues from inappropriate exposure to retinoic acid. *Cyp26a1*-null animals exhibit caudal agenesis and spina bifida, imperforate anus, agenesis of the caudal portions of the digestive and urogenital tracts, and malformed lumbosacral skeletal elements. This phenotype closely resembles the most severe form of caudal agenesis in humans. In mutational screening allowed to identify 6 SNPs, 4 of which (447C>G, 1134G>A, IVS1+10G>C, and IVS4+8AG>GA) are new **(De marco *et al*., 2006**). In addition, a novel 2-site haplotype consisting of the 2 intronic SNPs. Both single-locus and haplotype analyses revealed no association with increased risk for Caudal regression syndrome. The consequences of the 2 intronic polymorphisms on the mRNA splicing process was also investigated. Another Rat *et al*. (2006) gives the evidence for a functional genetic polymorphism of human retinoic acid metabolizing enzyme CYP26A1, an enzyme that may be involved in Spina bifida. Rat's represents the first description of a functional genetic polymorphism affecting the coding sequence of the human CYP26A1 gene. CYP26A1, together with CYP26B1 and CYP26C1, are key enzymes of all-trans retinoic acid (RA) inactivation and their specific and restricted expression in developing embryos participate in the fine tuning RA levels. The imbalance between the synthesis and degradation of RA during embryogenesis could contribute to malformations and developmental defects. The consequence of the 1-bp deletion identified in the coding sequence was investigated by an in vitro functional assay using COS-7 cells. A total of 7 polymorphisms were identified, comprising 1 nucleotide deletion in the coding sequence (g.3116delT) that results in a frame shift and consequently in the creation of a premature stop codon (**Rat *et al*., 2006**). The g.3116delT mutation is of particular interest because it was identified in a patient with spina bifida and likely encodes a truncated protein with no enzymatic activity, as demonstrated in vitro data.

Table 2.1: Types of CYP26A1 gene mutation reported in Human

Accession number	Mutation Type Deletion (^ codon number)	Number of Mutation	Phenotype	References
CX068379	**G^^{288}CAG_E5I5_GTGAGTagCAGCTTCAGA**	1	Reduced mRNA splicing efficiency	De Marco *et al.*,(2006)
CD067162	**AAGGAA^^{413}GAATtTAATCCTGAC**	1	Altered enzyme activity	Rat *et al.*, (2006).
	Splicing Mutation			
CS068378	**IVS2 ds +10 G-C**	1	Reduced mRNA splicing efficiency	De Marco *et al.*,(2006)

2.5 STUDY OF DRUG-RETINOID INTERACTIONS

CYP26A1 enzymes in general are often affected by many factors besides compounds that are considered to be their specific inducers or substrates. A limited number of studies have examined whether the oxidative metabolism of RA is affected by the use of bioactive compounds or drugs and whether agonists for other nuclear receptors, such as PPARs, might alter the induction of CYP26 gene expression by retinoids. The inhibitory effects of drugs on RA metabolism appears to be consistent with findings of lower serum concentration of at-RA and 13-*cis*-RA (**Fex and Felding, 1984**) and their metabolites, including 13-*cis*-4-oxo-RA (**Nau *et al.*, 1995**) in patients using anti convulsant drugs, such as phentoin, carbamazepine, and valporate, and antiepileptic drugs. Apparently, smoking and alcohol use do not significantly affect the RA-4-hydroxylation activity as measured in human liver specimens (**Van Heusden *et al.*, 1998; Nadin and Murray, 1999**), although chronic alcohol use influences the storage of vitamin A in the liver. However, these factors have not been specifically evaluated regarding effects on

the CYP26A1 family. RA-4-hydroxylation activity in hepatic microsomes was inhibited by more than 75% by parathion, a potent insecticide; by 50% by quinidine, a heart antiarrhythmic drug; and by 30% by ketoconazole, a general inhibitor of cytochrome P450 enzymes (**Nadin and Murray, 1999**). **Osanai *et al.* (2005)** demonstrated the expression of the Retinoic Acid-Metabolizing enzyme CYP26A1 limits programmed of cell death. However, the mechanisms by which Vitamin A depletion promotes tumorigenesis are poorly understood. In addition all-*trans*-retinoic acid (RA), the most active form of vitamin A metabolites, has been shown to limit carcinogenesis in animal models and to trigger programmed cell death (apoptosis) in certain types of tumor cells. On the other hand, various cell lines overexpressing CYP26A1, a cytochrome P450 enzyme specifically involved in the catabolic inactivation of RA, exhibit increased resistance to various apoptogenic factors, including death receptor ligands such as tumor necrosis factor-related apoptosis-inducing ligand. This resistance could be reversed by pretreatment with ketoconazole, a broad-spectrum inhibitor of cytochrome P450 enzymes. In addition, synthetic retinoids Am80 (4[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl]benzoicacid) and Am580 [4(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtamido)benzoic acid], which are resistant to CYP26A1 metabolism, can restore the sensitivity of these cells to apoptogens. A deficiency of vitamin A has long been associated with carcinogenesis, and several studies have shown an up-regulation of CYP26A1 mRNA expression in cancer cell types and tumors in vivo (**Shelton *et al*., 2006**). A concomitant decrease in the level of RA and increase in CYP26A1 mRNA has been shown in Barrett's associated dysplasia and esophagus adenocarcinoma, both in vitro and in vivo (**Chang *et al*., 2008**). CYP26A1 mRNA was elevated in 42% of primary breast cancer specimens (**Osanai *et al*., 2010)**. The enhanced expression of CYP26A1 suppressed cellular responses to anoikis and consequently resulted in promotion of anchorage-independent growth, whereas suppression of CYP26A1 by a specific siRNA reversed the oncogenicity, suggesting a direct link between RA signaling and tumorigenicity. Ischemia, as another factor, may also affect the expression of CYP26A1 but not CYP26B1 (**Tay *et al*., 2010**). The CYP26A1 mRNA transcript has been shown to be significantly lower in the livers of ischemic versus non ischemic individual livers.

2.6 INHIBITION OF THE CATALYTIC ACTIVITY OF CYP26 BY SPECIFIC COMPOUNDS

The anti proliferative properties of RA make it a drug of choice for the treatment of various disorders including leukemia, skin disorders such as psoriasis and acne, as well as an emerging therapy for conditions such as atherosclerosis (**Jacolot *et al.*, 1991; Fisher and Voorhees, 1996).** Because RA is a specific substrate and a potent inducer for both CYPA1 and CYPB1, the long-term use of RA may result in the development of resistance to RA therapy. Therefore, a better therapeutic approach for those disorders could be to inhibit the catalytic actions of CYP26 enzymes. Such an idea has led to the development of RA metabolism–blocking agents (RAMBAs) **(McCaffery and Sivas, 2009)**. A number of RAMBAs have been developed with high potency for inhibiting the metabolism of at-RA. Among them, for example, are compounds R116010 and R115866 (**Stoppie *et al.*, 2000)**. R116010 enhances the biological activity of at-RA and exhibited antitumor activity in a mouse mammary carcinoma model **(Van Heusden *et al.*, 2002**), whereas a single oral dose of R115866 in intact rats resulted in increases in endogenous tissue RA levels in plasma, skin, fat, kidney, and testis, and as a result R115866 (**Vincent *et al.*, 2006**) exerted retinoidal activities**. Altucci *et al. (*2007)** studied the therapeutic applications of at-RA and 13-cis-RA**.** While RA is synthesized in the body from dietary precursors, it may also be administered therapeutically. Both at-RA or tretinoin (Vesanoid®) and 13-cis-RA or isotretinoin (Accutane®) are administered clinically. Tretinoin is approved to induce cytodifferentiation and decrease proliferation of acute promyelocytic leukemia whereas isotretinoin is approved for the treatment of severe nodular acne that has been unresponsive to other forms of treatment. **Ocaya *et al.* (2007)** proposed that CYP26 inhibitor R115866 increases Retinoid Signaling in intimal Smooth Muscle Cells. According to them intimal smooth muscle cells (SMCs) are dedifferentiated SMCs that have a powerful ability to proliferate and migrate. This cell-type is responsible for the development of intimal hyperplasia after vascular angioplasty. Retinoids, especially all-trans retinoid acid, are known to regulate many processes activated at sites of vascular injury, including modulation of SMC phenotype and inhibition of SMC proliferation. Compound R115866 is shown to be effective in treating skin disorders, to potentially increase the endogenous level of

RA in the keratinocytes and epidermis (**Giltaire *et al.*, 2009; Pavez Lori`e *et al.*, 2009)**, and to increase retinoid signaling in intimal smooth muscle cells, which is postulated to offer potential new therapeutic ways to treat vascular proliferative disorders. In organotypic organ cultures of human skin, the addition of the cytochrome P450 inhibitors liarozole and talarozole in the presence of a very low concentration of RA (1 nM) increased CYP26A1 expression **(Pavez Lori`e *et al.*, 2009**), which may be due to the build up of RA to a sufficient level to activate nuclear receptors and thus increase trans activation of the CYP26A1 gene. To improve therapeutic outcomes synthetic retinoic acid analogs retinoic acid receptor (RAR) agonist and retinoic acid metabolizing blocking agent (RAMBAs) have been developed.