# STEROID BIOSYNTHESIS AND EMBRYONIC STEM CELL PROTEINS AS PUTATIVE PREDICTIVE BREAST CANCER BIOMARKER

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**By**

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**2008**

# RECOMMENDATION

This is to certify that Mr. Khagendra Koirala has completed this dissertation work entitled "**STEROID BIOSYNTHESIS AND EMBRYONIC STEM CELL PROTEINS AS PUTATIVE PREDICTIVE BREAST CANCER BIOMARKER"** as a partial fulfillment of M. Sc. Degree in Microbiology under our supervision. To our knowledge this thesis work has not been submitted for any other degree.

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**Khagendra Koirala**

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# ABSTRACT

Breast cancer is the second most common malignancy in women and is curable if diagnosed early. Sex steroid hormone (reproductive) is known to be involved in the breast cancer. Estrogen and androgen are known to be synthesized in breast carcinoma tissues and estrogen is known to be greatly contributing to the growth of breast carcinoma. Majority of breast cancer tissue express estrogen (ER) receptor, progesterone (PR) and androgen (AR) receptor and the level of expression of these receptors is important factor in prognosis and management of breast cancer. In this study expression of CYP17A, PP2A, CREB3L1, COX-2, and NANOG were investigated for identification of putative predicted biomarker for breast cancer susceptibility.

The expression of CYP17A and found to be expressed at around 70-60 kDa. In addition, a variant that has putative stop codon at 239 argine and transcribes 28 kDa protein was found. This is the first report for such allelic variant in breast cancer tissue. Most of the samples were heterozygous to this. Furthermore, it was found for the first time putative alternative transcript which is transcribed around 128 amino acid downstream to first methionine of normal transcript, and gives around 37 kDa protein.

PP2A is a regulatory protein that regulates the expression of steroidogenic genes. Expression of PP2A was looked into and found that the regulatory subunit is expressed in most of the samples. However, catalytic subunit to be expressed only in 71% of the samples indicating its regulatory role in expression of steroidogenic genes.

In addition, expression of CREB3L1, a CREB/ATF family transcription factor protein expressed in endoplasmic reticulum stress was observed. This is the first report for the expression of CREB3L1 in breast cancer. In addition, putative splicing variants of around 38 kDa and 20 kDa was observed. However the role of this protein in breast cancer from the present study was not explained but speculation was made that this protein might be involved in expression of protooncogene, or might have been involved in expression of variants of CYP17A and COX-2 among the proteins studied.

COX-2 expression and CYP19 (aromatase) expression has been found to be correlated in breast cancer. Aromatase is involved in biosynthesis of estrogen, and

estrogen has been found to be correlated with breast cancer. High expression of putative splicing variant of COX-2 was observed. In addition, it was found expression of COX-2 variants of 35 kDa and 25 kDa which could have been expressed from same gene by induction through signaling pathway. This is the first report indicating presence of putative COX-2 variants in human.

In order to clarify whether breast cancer tissue has pleopotency, expression of Embryonic Stem cell protein NANOG was looked. Expression of NANOG in breast cancer tissue was found. In addition, additional variant of 20 kDa that corresponds to the molecular size of Developmental pluripotency associated protein 3, which is also homeobox domain protein homolog of NANOG was found. This is in concordance to other reports that has reported expression of NANOG in breast cancer.

Keywords- breast cancer, biomarker, PP2A, CREB3L1, CYP17A, COX2A, NANOG

# TABLE OF CONTENTS


| **Title Page** | **PageNo** |
|---|---|
| | **i** |
| **Certificate of Approval** | **ii** |
| **Board of Examiners** | **iii** |
| **Acknowledgements** | **iv-v** |
| **Abstract** | **vi-vii** |
| **Table of Contents** | **vii xii** |
| **List of Abbreviations** | **xiii-xv** |
| **List of Tables** | **xvi** |
| **List of Figures** | **xvii** |
| **List of Photographs** | **xviii** |
| **Photographs** | **xix-xxi** |
| **List of Appendices** | **xxii- xxx** |
| 1. **CHAPTER I: INTRODUCTION** 1.1. Breast cancer | 1 |
| 1.2. Steroid metabolism and development of breast cancer | 2-9 |
| 1.3. Stem Cell Character of Breast Cancer: | 10- |
| 2. **CHAPTER II: OBJECTIVES** | **11** |
| 2.1 General objective | 12 |
| 3. **CHAPTER III: LITERATURE REVIEW** | |
| 4. 3.1 Protein phosphatase 2A (PP2A) and breast cancer | 13-16 |

3.2. Cyclic AMP responsive element binding protein 3L1 (CREB3L1, OASIS)

16-22

3.3. CYP17A1 22

3.4. COX2

25-27

3.5 NANOG

27-28

5. **CHAPTER IV: MATERIALS AND METHODS**

4.1 Materials

29

4.2 Methodology

29

4.2.1 Filling of informrd consent form

30

4.2.2 Sample collection and transport

30

4.2.3 Sample storage

30

4.2.4 Ripa buffer preparation

30

4.2.4.1 Incomplete ripa buffer preparation

30

4.2.4.2. Complete RIPA buffer preparation

31

4.2.5 Lysate preparation

31

4.2.6 Determination of protein

31

4.2.7 SDS PAGE Electrophrosis and Western blotting

32-38

4.2.8 Immunohistochemical analysis of breast cancer tissue\ 39-40

6. **CHAPTER V: RESULTS**

5.1 Clinical analysis of collected samples 41

5.2Commassie staining and band size marking 42

5.2 Expression profile of proteins observed in Western blotting 43-49

7. **CHAPTER VI: DISCUSSION AND CONCLUSION**

6.1 Discussion 50-57

6.2 Conclusion 57-60

8. **CHAPTER VII: SUMMARY AND RECOMMENDATIONS**

7.1 Summary 61-62

7.2 Recommendation 62

8. **REFERENCES** 63-85

# LIST OF TABLES

Table-1 Clinical data of patients with breast cancer
Table-2 Protein phosphatase 2A (around 65kDa)
Table-3 Protein phosphatase 2A(around 37kDa)
Table-4 cAMP element response binding protein 55 kDa
Table-5 cAMP element response binding protein for 37 kDa
Table-6 cAMP element response binding protein for 45 kDa
Table-7 cAMP element response binding protein for 80 kDa
Table-8 Cytochrome 17A for around 75kDa
Table-9 Cytochrome 17A for between 37kDa and 25kDa
Table-10 COX2, above 50kDa
Table-11 COX2 between 37 and 25kDa
Table-12 NANOG around 20 kDa
Table-13 NANOG for 75kDa
Table-14 NANOG between 50 and 35kDa

# LIST OF FIGURES

Fig 1 Steps and different enzymes involved in steroid biosyntheis.

Fig 2 Proposed pathway of steroid biosynthesis regulation

Fig 3 Hormone Synthesis Pathway from Cholesterol

Fig 4 The origin of estrogenic Steroids in postmenopausal women with hormone dependent breast cancer

Fig 5 Schematic representation of major PP2A-dependent pathways that are targeted by SV40 ST to promote cell transformation

Fig 6 cAMP pathway from the cell surface to the nucleus

Fig **7** Modular structure of the transcription factors of the CREB family

Fig 8 cAMP pathway activation in various causes of Cushing's syndrome

Fig 9 CYP17A1 mediated monooxygenase enzymatic pathway

Fig 10 Flow chart of methods

Fig 11 Protein size labeling using standard marker

Fig 12 Commassie staining of transferred lysates of samples.

Fig 13 Scanned figure of X-ray film after Western Bloting using anti PP2A antibody

Fig 14 Expression profile of CREB3L1 in breast cancer, placenta and umbilical cord

Fig 15 Expression profile of CYP17A in breast cancer, placenta and umbilical cord tissue

Fig 16 Expression profile of COX2 in breast cancer, placenta and umbilical cord tissue

Fig 17 Expression profile of NANOG in breast cancer, placenta and umbilical cord

# LIST OF ABBREVIATION

| | |
|---|---|
| APS | Ammonium persulphate |
| 3ΔHSD | Hydroxysteroid dehydrogenase, delta isomerase |
| BC | Blue carrier protein |
| BSA | Bovine serum albumin |
| CLCA1 | Chloride channel, calcium-activated, 1 |
| CREB | cAMP response element (CRE)-binding protein |
| COX-2 | Cyclooxygenase-2 |
| CYP | Cytochrome P450 |
| DAZL | Deleted in azoospermia like |
| DCIS | Ductal carcinoma in situ (DCIS) |
| DES | Diethylstillbestril |
| EBO | Everest Biotech number |
| ELISA | Enzyme linked immunoassay |
| ER | Estrogen receptor |
| ERSE | ER stress responsive element |
| FCA | Freund's complete adjuvant |
| FFTP | First full term pregnancy |
| FIA | Freund's incomplete adjuvant |
| GDF3 | Growth/Differentiation Factor |
| HAD | Human adenoid tissue |
| HMU | Human muscle |
| HLI | Human liver |
| HLU | Human lungs |
| hESC | Human embryonic stem cell |
| IBC | Inflammatory breast cancer |
| Ig | Immunogloblulin |
| IDC | Invasive (infiltrating) ductal carcinoma |
| ILC | Invasive (infiltrating) lobular carcinoma |
| KLH | Keyhole limpet hemocyanin |
| MAPK | Mitogen activated protein kinase |
| MIF | Migration inhibitory factor |

| | |
|---|---|
| MES | 2-N-morpholino ethane suphonic acid |
| MLI | Mouse liver |
| Na2 EDTA | Ethylene diaminetetraacetic acid, disodium salt Na2 |
| NFDM- | Non-fat dry milk |
| NPI | Nottingham prognostic index |
| NSAIDs | Nonsteroidal anti-inflammatory drugs |
| OASIS | Old astrocyte specifically induced substance |
| PBS | Phosphate buffer saline |
| PGHS2 | Prostaglandin-endoperoxide synthase-2 |
| PKA | Protein kinase A |
| PMSF | Phenylmethylsulphonylfluoride |
| PNPP | p-nitrophenyl phosphate |
| PP | Phosphoprotein phosphatase |
| PGC | Primordial germ cell |
| RBR | Rat brain |
| RIP | Regulated intramembrane proteolysis |
| RTE | Rat testis |
| S1P | Site-1 protease |
| S2P | Site-2 protease |
| SREBP-2 | Sterol regulatory element binding protein 2 |
| SCAP | SREBP cleavage-activating protein |
| TBST | Tris-buffer saline tween-20 |

# LIST OF PHOTOGRAPHS

Photograph-1 ER positive result of cancerous tissue seen under microscope

Photograph-2 ER negative result of cancerous tissue seen under microscope

Photograph-3 PR positive result of cancerous tissue seen under microscope

Photograph- 4 PR negative result of cancerous tissue seen under microscope

# CHAPTER-I

## 1. INTTRODUCTION

### 1.1 Breast cancer

Breast cancer is a malignant (cancerous) tumor that starts from cells of the breast. The disease occurs mostly in women, but men can get breast cancer too. A woman's breast is made up of glands that make breast milk (lobules), ducts (small tubes that carry milk from the lobules to the nipple), fatty and connective tissue, blood vessels, and lymph vessels. Most breast cancers begin in the cells that line the ducts (ductal cancer), some begin in the lobules (lobular cancer), and the rest in other tissues. (Susan et al., 1999)

Although there has not been single or dominant etiological factor for progression of breast cancer, but several factors are considered as high risk. Gender, family history, earlier breast radiation, treatment with DES (diethylstillbestril), being overweight or obese, lack of exercises, pollution, personal history of breast cancer, early menarche and late menopause, delayed pregnancy, intake of birth control pills, post menopause hormone replacement, breast feeding habit, alcohol consumption etc. Intensive researches have been carried out in relation between steroid hormones and breast cancer. Steroids are lipids without glycerol and their basic structure is four interlocking rings of carbon atoms. Mammalian steroid hormones can be grouped into five groups by the receptor to which they bind: glucocorticoids, mineralocorticoids, androgens, oestrogens and progestagen. The precursor of all the steroidal hormone is cholesterol and differences in their respective biological activities between steroid hormones is due to the small alterations or substitution in the cholesterol backbone. Persistently increased blood levels of estrogen are associated with an increased risk of breast cancer, as are increased levels of the androgens androstenedione and testosterone (which can be directly converted by aromatase to the estrogens estrone and estradiol, respectively, Fig 3, Breast Cancer research treat, 2007)

### 1.2 Steroid metabolism and development of breast cancer

Steroid hormone level has shown correlation with breast cancer progression. The contribution of the reproductive hormone, estrogen, to the development of breast cancer has been well documented in epidemiological and molecular and cell biology studies (Rock et al., 2007). Estrogen triggers cell growth and tumor promotion by binding to the estrogen receptor (ER) in the nucleus, leading directly to expression of many target gene involved in cell cycle progression (Mao C et al., 2007).

Cytochrome P450 (CYP) is a family of heme containing monooxygenase protein involved in the metabolism of huge number of exogenous and endogenous compound. The CYP super family is classified into families and subfamilies based on amino acid similarity, and 14 families have been reported in mammals. CYPs are involved in synthesis of steroid and bile acids and hydroxylation of fatty acids, or elimination of xenobiotics and steroids from the body. In human there are around 53 isoforms of and about 6, CYP11A1, CYP17A1, CYP21A1, CYP11B1 and CYP11B2, CYP19 (aromatase), are involved in hormone biosynthesis including oestrogen (Wilson et al., 2004).

By side chain cleavage CYP 11A1 converts cholesterol into prenelonone, a precursor for all steroid hormone synthesis. Pregnelonone is hydorxylated by CYP17A1 to 17-hydroxypregnelonone, which is further hydroxylated to give dehydroepiandrosterone. Pregnelonone is also metabolized by 3-beta hydroxysteroid dehydrogenase, delta isomerase, type 2 (3βHSD) to progesterone. Progesterone is further metabolized into 17-hydroxyprogesterone by CYP17A1, or 3βHSD mediated metabolism of 17-hydroxypregnelonone. CYP 17A further metabolizes 17-hydroxypregesterone to give andrestenerone, which is also synthesized from metabolism of dehydroepiandrosterone by 3βHSD. CYP21A1 metabolizes progesterone to deoxycorticosterone, and 17-hydroxyprogesterone to 11-deoxycortisone. Deoxycorticosterone is metabolized to corticosterone by CYP11B1 which is further metabolized to aldosterone by CYP11B2. CYP11B1 metabolizes 11-deoxycortisone into cortisol. Thus it is evident that CYP family plays pivotal role in steroid biosynthesis.

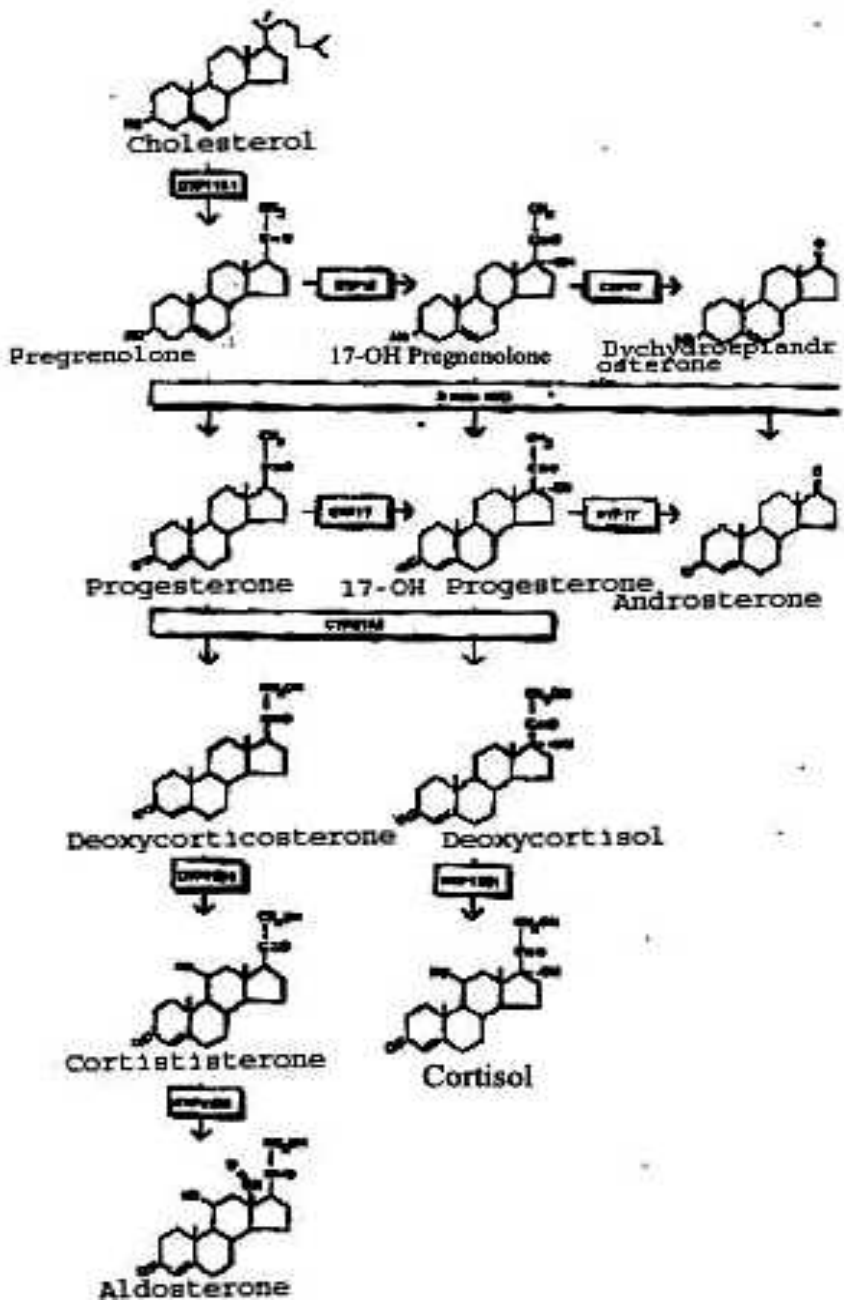


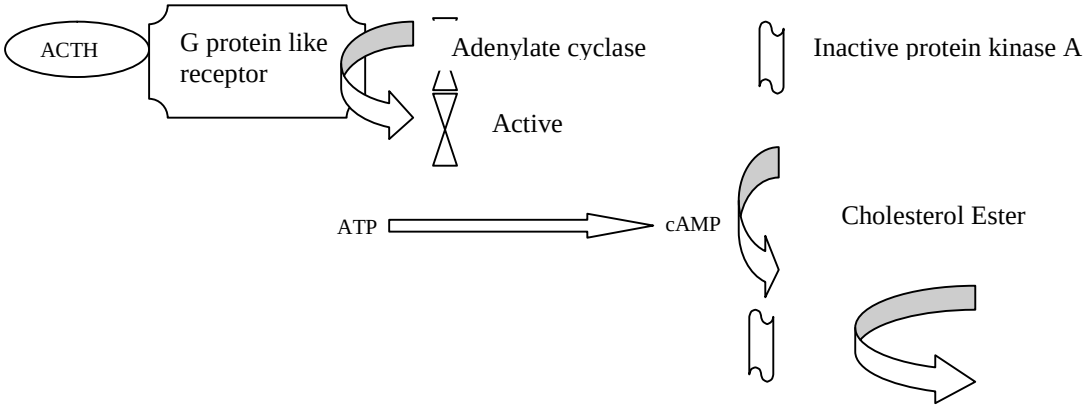

Fig 1 Steps and different enzymes involved in steroid biosynthesis (Wilson et al., 2004)

In a case control studies Yaspan et al., (2007) have found that variants designating a haplotype encompassing the gene promoter are significantly associated with both increased expression and increased breast cancer risk. Among genes controlling endogenous estrogen metabolism, CYP11A1 harbors common variants that may influence expression to significantly modify risk of breast cancer.

A pentanucleotide tandem repeat [(TAAAA)n] polymorphism in the 5' of the CYP11A gene has been reported to be related to the risk of polycystic ovary syndrome, an inherited endocrine disorder characterized by hyperandrogenemia. In a case control study Zheng et al., (2004) found had of high risk to breast cancer, 1.5 times for heterozygous and 2.9 times for homozygous.

The cAMP-dependent protein kinase A (PKA) is known to regulate a variety of cellular functions. Various extra cellular signals converge on this signaling pathway through ligand binding to G-protein-coupled receptors. Steroidal homeostasis is maintained by adrenal cortex, which on depletion of steroid level in blood synthesizes ACTH that on binding to G-protein coupled receptor activates adenosine cyclase generating cAMP pool which then activates PKA dependent signaling cascade for expression of steroidogenic genes. It has been reported that PKA is involved in cell growth and differentiation. Over expression of PKA has been found to be correlated with worse clinical pathological features and prognosis in terms of recurrence and overall survival in breast cancer patients.

PKA and other kinases (CaMK, MAPK/p90rsk, PKC, p38, PKB/Akt) phosphorylate Ser133 in the kinase-inducible domain of CREB and activate CREB-dependent transcription The cAMP response element (CRE)-binding protein (CREB) is a 43-kDa transcription factor, which controls gene transcription in the basal state and in response to diverse signals initiated by hormones and neurotransmitters.. Many reports have defined a role of CREB in proliferation, differentiation, and migration. Synergistic interaction between factors bound to different sites within a transcriptional control region is supported by the work of Courey et al., (1989). One of the proteins that regulate the expression of steroidogenic genes is PP2A.



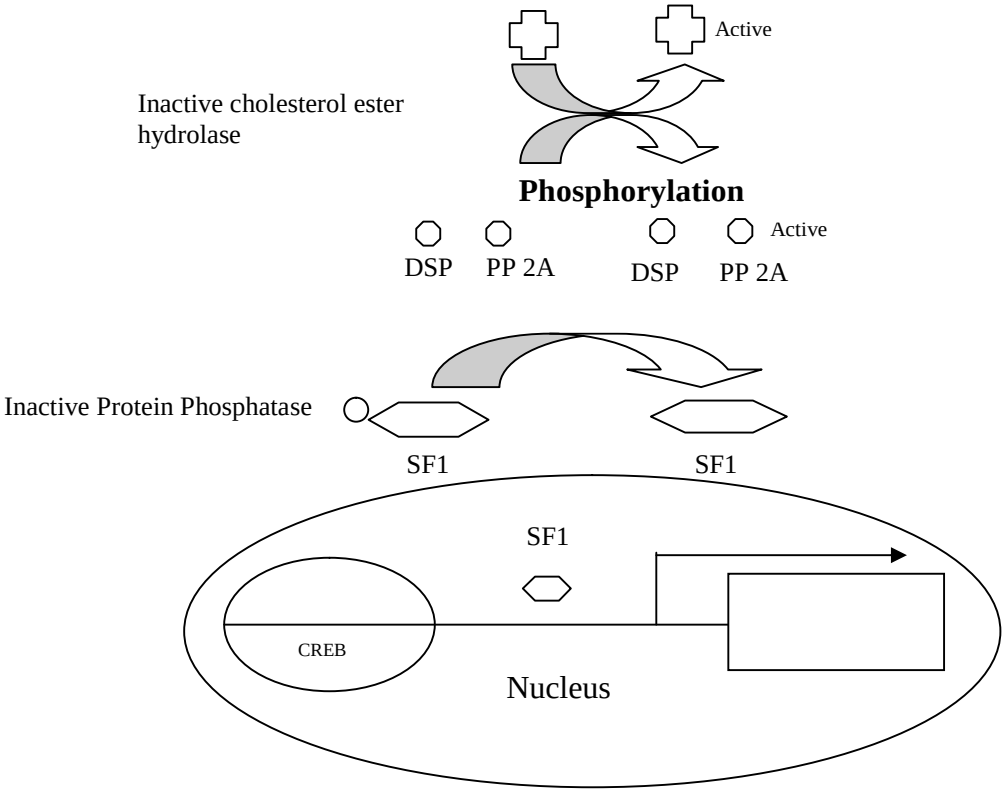



Figure 2 Proposed pathway of steroid biosynthesis regulation

A variety of cellular processes such as differentiation, lipid metabolism, and response to unfolded proteins are mediated through regulated intramembrane proteolysis (RIP) that allows for a rapid response to the regulatory signals (Brown et al., 2000). One of the characterized RIP protein is the sterol regulatory element binding protein 2 (SREBP-2) of the SREBP family of transcription factors that regulate genes involved in the cholesterol and fatty acid metabolism (Brown and Goldstein, 1999). Kondo et al., (2005) had identified a novel ER stress transducer, OASIS (old astrocyte specifically induced substance) or CREB3L1, a basic leucine zipper (bZIP) transcription factor of the cAMP response-element binding protein (CREB)/ATF family with a transmembrane domain that allows it to associate with the ER.

Taking into consideration the similarity of CREB3L1 and SREBP-2 as RIP, CREB3L1 having distinct character to CREB, steroidogenic genes having CREB binding domain in its promoter regions, RIP proteins being involved in cellular response for differentiation, lipid metabolism we hypothesized that the CREB3L1 might be expressed in breast cancer in regulation of expression of steroidogenic genes

converting cholesterol to different steroids possibly binding to sterol-regulating elements. The expression pattern of CREB3L1 was studied.

Overall pathway of steroid biosynthesis and role of different enzymes directly involved is summarized in Fig. 3, 4.

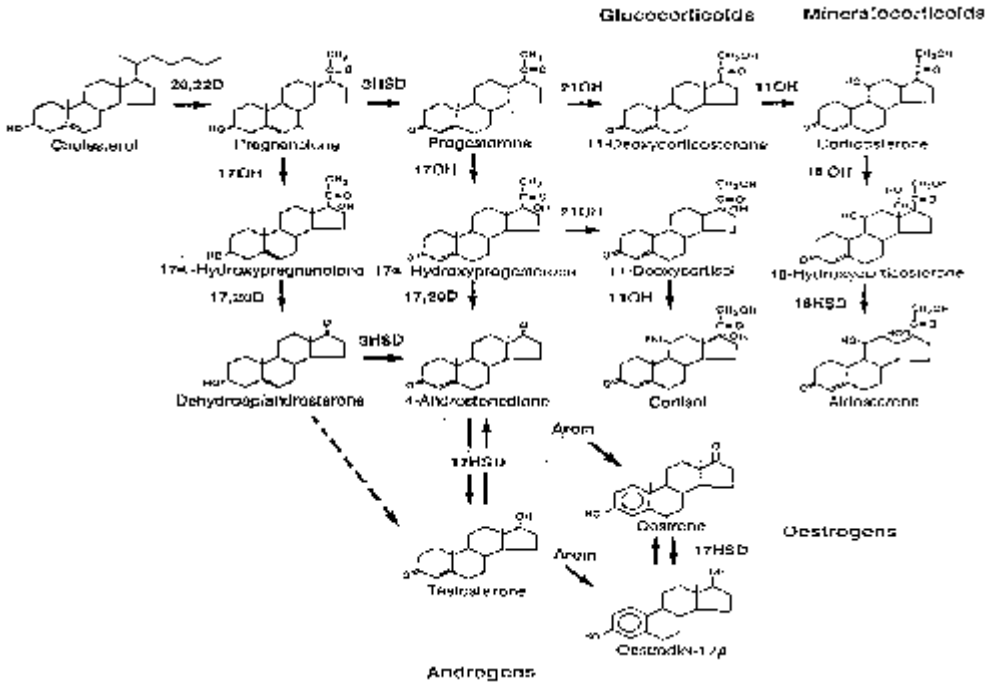



Fig 3 Hormone synthesis pathway from cholesterol (Wilson et al., 2004)

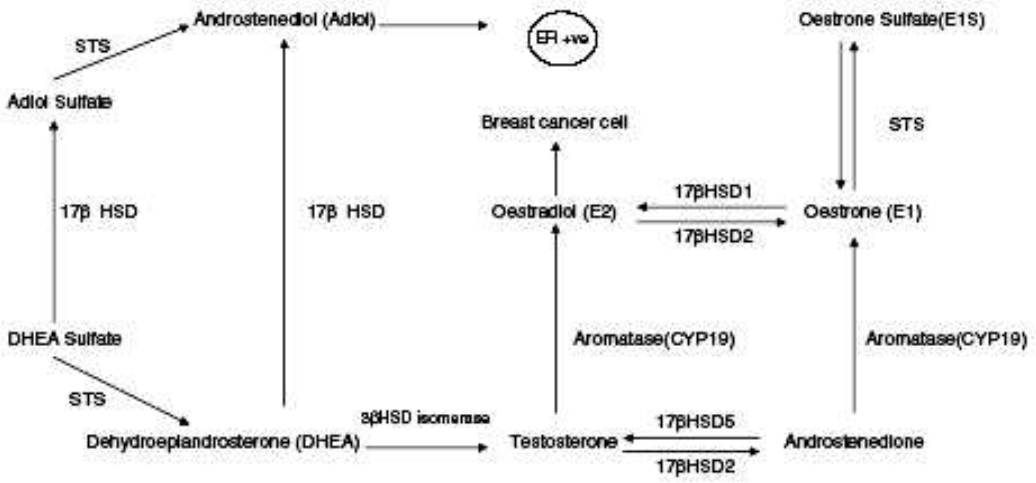



Fig 4 The origin of estrogenic Steroids in postmenopausal women with hormone dependent breast cancer

HSD-Hydroxysteroidhydrogenase; STS-Steroid Sulphate; EST-Estrogen Sulphotransferase; Source-Breast Cancer research treat, 2007

Cyclooxygenase-2 (COX-2), the enzyme that converts arachidonic acid to prostaglandin H2, is expressed in normal brain and kidney, activated macrophages, synoviocytes during inflammation, and malignant epithelial cells. COX-2 expression is stimulated by a number of inflammatory cytokines, growth factors, oncogenes, lipopolysaccharides, and tumor promoters. There is evidence that COX-2 plays a key role in tumorigenesis through stimulating epithelial cell proliferation, inhibiting apoptosis, stimulating angiogenesis, enhancing cell invasiveness, mediating immune suppression, and by increasing the production of mutagens. Results of several studies using mouse models of colon cancer and the results of clinical trials have shown COX-2 to be a useful target for the prevention and treatment of colon cancer. Studies with several other epithelial cancers involving different organ sites, e.g., breast, prostate, bladder, lung, and pancreas, suggest that COX-2 plays an important role in the pathogenesis of these cancers. Studies from mouse models of mammary tumorigenesis and from human breast cancer cell lines provide evidence that COX-2 over expression plays an important role in the pathogenesis of malignant breast cancer in humans (Singh et al., 2002).

Singh et al., (2005) found over expression of COX-2 protein.. They further observed that pro-urokinase plasminogen activator (pro-uPA) expression was significantly higher (approximately 5-fold) in transfected cells. It is known that an increase in cell migration and invasion can be brought about by cytoskeletal alterations and basement membrane degradation due to increased expression of pro-uPA. They concluded that COX-2 over expression in human breast cancer cells enhances cell motility and invasiveness thus suggesting a mechanism of COX-2 mediated metastasis.

COX-2 is known to increase intracellular cAMP and estrogen production in malignant breast tissue. The aromatase enzyme complex is responsible for local production of estrogens in breast cancer (Fig. 3). Increasing evidence supports a role for COX-2 in upregulation of aromatase activity. Salhab et al., (2007) found COX-2/CK19 of both benign and malignant tissues were positively correlated with aromatase/CK19 transcript levels and concluded that COX-2 is an upregulator of aromatase in breast tissue. It is evident that the risk of developing breast cancer is strongly correlated with

the overall exposure to estrogen and most tumours are more or less dependent on estrogen for their growth.

Aromatase expression and enzyme activity in breast cancer patients is greater in or near the tumor tissue compared with the normal breast tissue. Complex regulation of aromatase expression in human tissues involves alternative promoter sites that provide tissue-specific control. Strong association between aromatase (CYP19) gene expression and the expression of cyclooxygenase (COX) genes has been reported and the nonsteroidal anti-inflammatory drugs (NSAIDs) and COX selective inhibitors can suppress CYP19 gene expression and decrease aromatase activity (Brueggemeier et al., 2007) indicating that COX-2 can influence biosynthesis of estrogens. Brueggemeier et al., (2007) found that treatment of breast cancer cell lines with siCOX-2 decreased expression of COX-2 mRNA, and also suppression of CYP19 mRNA. Likewise, treatment of siCYP129 completely masked aromatase activity, indicating that suppression of COX-2 expression down regulates expression of aromatase thus decreasing the oestrogen synthesis.

In addition, inflammation and hormonal signalling is known to induce cyclooxygenase 2 (COX2) expressions in solid tumours. COX2 expression is linked to neovascularization and tumour growth. Witton et al., (2004) found that 21.2% of breast cancer showed higher level of expression of COX2 in epithelial cells than in normal.

In the present study, gene expression profile of COX-2 was performed taking into consideration the association between COX-2 and aromatase, its association with estrogen biosynthesis, and breast cancer.

### 1.3 Stem cell character of breast cancer

Expressions of pluripotency proteins are observed in human embryonic stem cell, (hESC), primordial germ cell (PGC) and carcinoma *in situ* (CIS) and embryonic carcinoma (EC). (Perrett et al., 2006; Rajpert-De Meyts, 2006; Western et al., 2005). NANOG is a transcription factor that plays key roles in the self-renewal and maintenance of pluripotency in human embryonic stem (ES) cells. Human embryonic stem cells express genes common to primordial germ cells (PGCs), and they express GDF3, STELLAR, NANOG proteins located in chromosome 12p.They found that

OCT4, STELLAR, NANOG and GDF3 mRNA is expressed in breast cancer cell line MCF-7 and detected NANOG in breast carcinoma, but not DAZL and VASA. This indicated that breast cancer resembles to seminoma with primordial germ cell character, but they are not of germ cell origin. These results indicated that stem cell genes may either play a direct role in different types of carcinoma progression or serve as valuable markers of tumorigenesis (Ezeh et al., 2005). Among Nanog's 11 pseudogenes (Hart et al., 2004). NANOGP8 theoretically could be a retrogene, but was considered unlikely as it has not been identified in any expressed sequence tags (ESTs). Zhang et al., (2006) found that NANOGP8 is expressed in several cancer cell lines and in all cancer tissues tested. They found that it is highly homologous to NANOG. The protein expressed in *Escherihia coli* and some other cancer cell lines was detected by anti-NANOG antibody. Cell proliferation was found to be promoted on expression of NANOG in NIH3T3 cells. These results indicate that NANOG, NANOGP8 may play important roles in tumorigenesis by enhancing cell proliferation. Expression of NANOG was not found to be altered in murine ESCs under stress, however, the cells showed apoptosis induced by etoposide, heat shock or UV exposure. However, increased expression level of OCT-4 was found in such condition. Further analysis revealed that survivin, a protein involved in anti-apoptosis, was found to be decreased. But in Oct-4 knock-out cells the morphology and expression level of NANOG did not alter, however, expression level of survivin. In breast cancer, STAT3 regulates expression of survivin and down regulated OCT-4 has been associated with decreased phoshphorylation of STAT3. This results indicate that NANOG helps in proliferation of breast cancer cells, and OCT-4 mediates in decreased apoptosis, most probably by preventing desphosphorylation of STAT3 (Guo et al., 2007) (Fig. 5). With these reports and pleopotency of breast cancer expression profile of NANOG was investigated.

Central to steroid biosynthesis, we looked into expression profile of protein involved in regulation of expression of steroidogenic genes, protein that may influence the metabolism of hormone, and protein that may give breast cancer pleopotency and rapid growth. The proteins investigated were CYP17A1, PP2A, CREB3L1, COX-2 and NANOG.

Since India and Nepal are culturally Hindu Philosophy dominated it is hard to get control tissue sample for comparative study. Expression profile of human placenta and umbilical cord from healthy control laboring mother has been used as control tissue.

# CHAPTER-II

## 2. OBJECTIVES

### 2.1 General objective

To identify putative biomarker of breast cancer susceptibility by comparative study on expression profiling of proteins involved in up-regulation, gene expression, biosynthesis, and metabolism of steroid hormones and embryonic stem cell modulator proteins.

### 2.2 Specific objectives

1. To study the expression patterns of following protein by western blotting in breast cancer tissue.

i. PP2A
ii. CREB
iii. CYP17A
iv. COX-2
v. NANOG

2. To compare these expressions with ER, PR level in breast cancer tissue and its clinical correlation.

# CHAPTER III

## 3. Literature Review

### 3.1 Protein phosphatase 2A (PP2A) and breast cancer

Protein phosphorylation, a posttranslational modification step controlling many diverse cellular functions, is dependent on the opposing actions of protein kinases and protein phosphatases. Loss of heterozygosity (LOH) of chromosome 11 at 11q22-q24 has been associated with lung, colon, breast, cervical, head and neck, and ovarian cancers, as well as melanoma (Arai et al., 1996). In nude mice, introduction of a normal chromosome 11, or a derivative t(X;11) chromosome containing 11pter-q23 reverses the tumorigenic potential of several types of cancer cells and Wilms tumor indicating that this region, centromeric to the t(X;11) breakpoint at 11q23, has one or more tumor suppressor genes.

Using radiation hybrid map of chromosome 11 and from the NCBI transcript map Wang et al., (1998) identified over 100 candidate genes and expressed sequenced tags (ESTs) between D11S1647 and D11S1987 markers as high frequency of LOH observed in lung cancer cells. One of the EST sequences (M65254) corresponded to a subunit of the serine/threonine protein phosphatase 2A (PP2A).

PP2A is one of the four major eukaryotic cytosolic protein phosphatases responsible for the dephosphorylation of serine and threonine residues of proteins. Although all four protein phosphatases, PP1, PP2A, PP2B, and PP2C, have overlapping substrate specificities in vitro, however, they can be distinguished by the use of inhibitors and by dependence on metal ions. The type 2 phosphatases can be distinguished by regulation of their activity: PP2A activity is independent of metal ions, PP2B is activated by $Ca^{2+}$/calmodulin, and PP2C is activated by $Mg^{2+}$ (Cohen and Cohen, 1989). The mammalian PP2A enzyme can be isolated as a catalytic subunit of 36 kD complexed to1 regulatory subunit of 65 kD and to another regulatory subunit of

varying molecular mass, depending on the tissue and the separation technique used (Cohen et al., 1989 and Chowdhury et al., 2005).

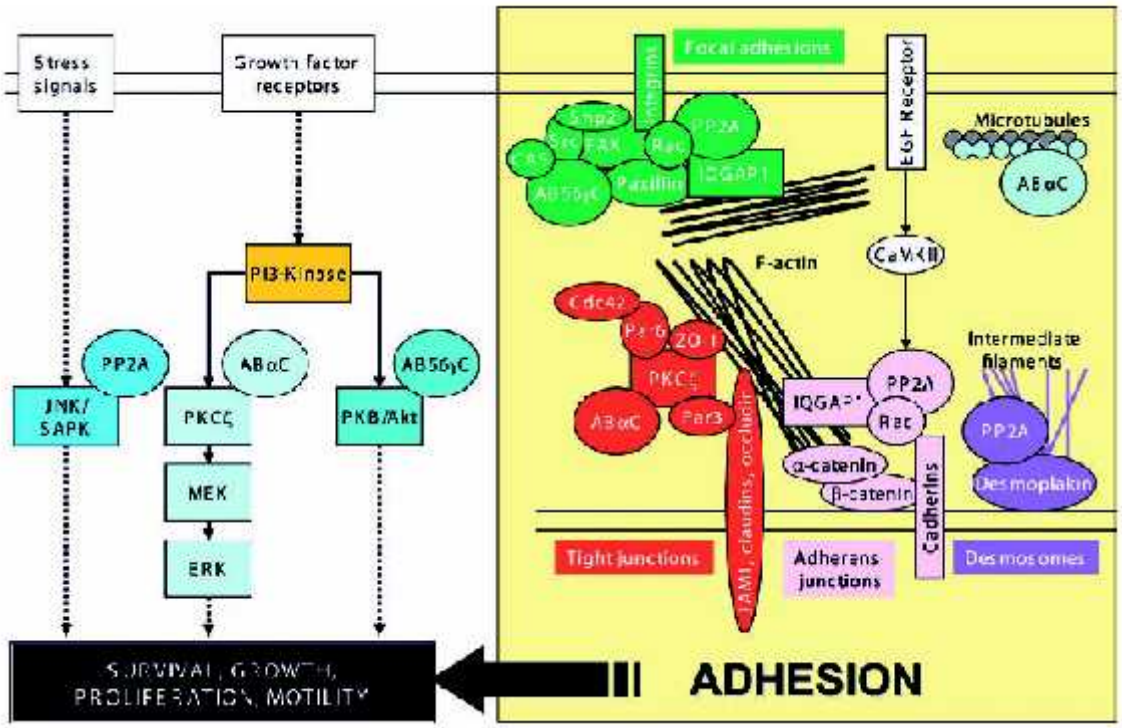



Fig 5 Schematic representation of major PP2A-dependent pathways that are targeted by SV40 ST to promote cell transformation. PP2A enzymes are essential regulators of intertwined signaling cascades and multi-protein adhesion complexes that converge to regulate cell polarity, growth, survival and proliferation. These PP2A-dependent pathways become altered in cancer, resulting in increased cell motility and invasiveness.

In tumorogenesis PP2A may function as either a tumor promoter or tumor suppressor, depending on the cell type or the transforming agent. It is found to be involved in the regulation of most major metabolic pathways, as well as translation, transcription, and control of transition from G2 to the M phase of the cell cycle. This is an important regulatory enzyme that downregulates the mitogen-activated protein kinase (MAPK) cascade, relays signals for cell proliferation, and appears to be linked to carcinogenesis.

The A subunit of protein phosphatase 2A (PP2A) consists of 15 nonidentical repeats. The catalytic C subunit binds to C-terminal repeats 11 - 15 and regulatory B subunits

bind to N-terminal repeats 1 - 10. Recently, four cancer-associated mutants of the A-alpha subunit have been described: Glu64-->Asp in lung carcinoma, Glu64-->Gly in breast carcinoma, Arg418-->Trp in melanoma, and Delta171 - 589 in breast carcinoma (Ruediger et al., 2001)

Most cancer cells have increased levels of telomerase activity implicated in cell immortalization. Activation of telomerase, a ribonucleoprotein complex, catalyzes the elongation of the ends of mammalian chromosomal DNA (telomeres), the length of which regulates cell proliferation. Currently, how telomerase is regulated in cancer is not yet established. But the findings have suggested that protein phosphorylation reversibly regulates the function of telomerase and that PP2A is a telomerase inhibitory factor in the nucleus of human breast cancer cells (Li H et al., 2004).

Suzuki et al., (2006) reported that both human breast cancer and MCF-7 cells were deficient in expression of the PP2A-A protein. Immunohistochemical protein array analysis revealed that PP2A was expressed in 43% of the normal-carcinoma where as 96% human breast tissue expressed. However, expression of E-cadherin in MCF-7 cells was 1.5-fold higher than that in human mammary epithelial (HME) cells with 80% of E-cadherin endocytosed and incompletely anchored to F-actin indicating that the dysfunction of E-cadherin due to its endocytosis may occur in some proportion of human breast carcinomas in which the PP2A-A protein is lost or significantly reduced.

In cell-mediated adhesions in breast cancer spheroids study with several breast cancer cell lines Ivascu and Kubbies, (2007) identified that E-cadherin mediates the spontaneous formation of spheroids in MCF7, BT-474, T-47D and MDA-MB-361 cells, whereas N-cadherin is responsible for tight packing of MDA-MB-435S cells. However, in MDA-MB-231 and SK-BR-3 cells formation of spheroids is mediated primarily by the collagen I/integrin β1 interaction with no cadherin involvement. This can primarily explain report of Suzuki et al., 2002 where E-cadherins is highly expressed. Taking into account of Suzuki et al and Ivascu and Kubbies report, homophilic E-cadherin and integrin beta1/collagen I interaction establishes spheroids in MDA-MB-468 cells. These findings indicate that an evolutionary diverse and complex pattern of interacting cell surface proteins exists in breast cancer cells that determines the 3D growth characteristic in vitro, thereby influencing small molecule

or antibody permeation in preclinical in vitro and in vivo tumor models. (Ivascu et al., 2007)

### 3.2. Cyclic AMP responsive element binding protein 3L1 (CREB3L1, OASIS)

Cyclic AMP (cAMP) second messenger pathways provide a chief means by which cellular growth, differentiation, and function can be influenced by extracellular signals. Activation of the cAMP pathway by seven domains transmembrane receptors coupled to the G□s protein leads to increased cAMP intracellular levels. The main target of cAMP is the cAMP-dependent protein kinase (PKA). As shown in figure the inactive state PKA is an heterotetramer of paired regulatory and catalytic subunit (C) and is located mainly in the cytoplasm. After activation by cAMP the catalytic subunit dissociates from the regulatory subunit and diffuses into the nucleus. Subsequently, the free nuclear C subunit of PKA phosphorylates the transcription factor CREB at 133 serine residue.

Following hormonal stimulation of a neuroendocrine cell, for example, increased cAMP levels activate cAMP-dependent protein kinase A, which phosphorylates one or more DNA-binding proteins. All cAMP-responsive gene promoters have in common an 8-base enhancer known as the cAMP-response element (CRE) containing a conserved core sequence, 5-prime-TGACG-3-prime, first described in the somatostatin gene by Montminy et al., (1986). Montminy and Bilezikjian (1987) purified a 43-kD nuclear phosphoprotein, which binds to CRE with high affinity.

After binding to their specific G protein–coupled transmembrane receptors peptide hormones act at the cellular level by stimulation of the cAMP signaling pathway. Alterations of the cAMP pathway have been implicated in several endocrine diseases. One major cellular effect of the cAMP cascade activation is the stimulation of transcription after phosphorylation of nuclear factors by the cAMP dependent protein kinase, PKA.

The transcription factor CREB is the best characterized nuclear protein that mediates stimulation of transcription by cAMP. The important role of CREB in the development and differentiation of various endocrine tissues has been demonstrated *in*

*vitro* and *in vivo*. In endocrine tumors alterations of CREB and related transcription factors have been reported. Rosenberg, et.al detected the physiological role of the transcription factors of the CREB family in endocrine tissues and their alterations in endocrine tumors. Expression of the CREB gene is ubiquitous.

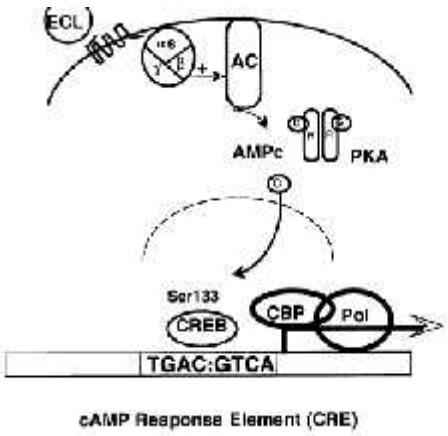



Fig 6 cAMP pathway from the cell surface to the nucleus. The extracellularligand (ECL) binds to its specific seven domains transmembrane receptors, leading to activation of an heterotrimeric Gs protein. Activation of the Gs protein leads to dissociation of its delta □subunit from the □□complex, stimulating adenylyl cyclase (AC) activity and therefore cAMP production. The PKA is activated after binding of cAMP. The free catalytic subunit (C) then goes into the nucleus and phosphorylates CREB at Ser133. Phospho-CREB binds CBP (CREB binding protein) that interacts with the polymerase (Pol), leading to transactivation. (Rosenberg et al., 2002)

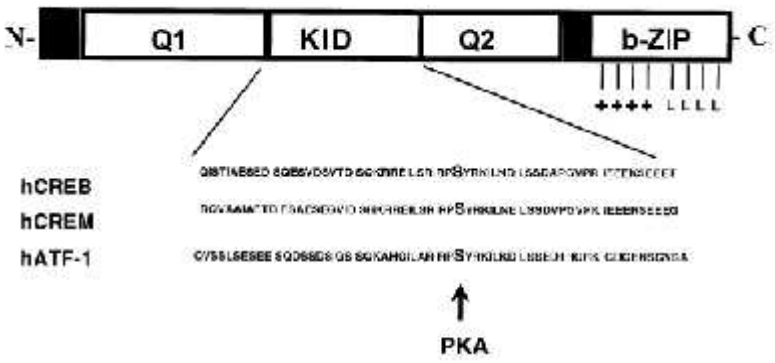



Fig 7 Modular structure of the transcription factors of the CREB family. Q1 and Q2 are the glutamine-rich domains of CREB. The KID is the kinase-inducible domain that is highly homologous between CREB, CREM, and ATF-1 as shown in the figure and contains the serine residue phosphorylated by PKA. The b-ZIP domain at the C

terminus binds DNA and the partner of the homo- or heterodimer of transcription factors. (Rosenberg et al., 2002)

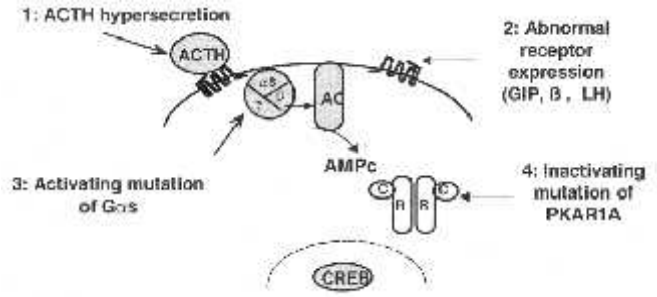



Fig 8 cAMP pathway activation in various causes of Cushing's syndrome. The figure shows alterations of the cAMP pathway at the extracellular, as well as the intracellar, level, in various forms of Cushing's syndrome associated with adrenal hyperplasia, adrenal tumors, macro- or micronodular adrenocortical hyperplasia. It is important to note that are associated with benign tumors. 1**.** Eutopic (Cushing's disease) or ectopic ACTH secretion. 2**.** Abnormal expression of membrane receptor: GIP (gastric-inhibitory peptide) receptor, -adrenergic receptor, LH receptor. 3**.** Activating mutation of the subunit of the Gs protein, termed oncogene Gsp in McCune-Albright syndrome. 4**.** Inactivating mutation of the PRKAR1A gene in Carney complex. (Rosenberg et al., 2002)

As explained in figure, several diseases with endocrine gland hyperplasia or tumor and hormone hyper-secretion can be secondary-that is, caused by activation of the extra cellular signal that stimulates the cAMP pathway in a given tissue.

Honma et al., (1999) identified and characterized mouse CREB3L1, (named OASIS, for old astrocyte specifically-induced substance) expressed in long-term cultured mouse astrocytes, or 'old astrocytes (OA)'. The OASIS gene encoded a putative transcription factor belonging to the cyclic AMP responsive element binding protein/activating transcription factor (CREB/ATF) gene family, with homology to box B-binding factor-2 (BBF-2), a Drosophila transcription factor. After birth at P14, the protein is detected at low levels in the cerebral cortex, hippocampus and thalamus. In the adult brain, expression becomes weaker.

Hikake et al., (2003) found that in mouse the protein is primarily expressed in the cartilage, tooth germs and salivary gland in the embryo. During differentiation stage the protein is expressed in the inner enamel epithelium during the cap and bell stages in the preodontoblasts and during early secretory stage in the differentiating odontoblasts. They found that proteoglycans (versican, decorin, biglycan, glypican, syndecan-1, and syndecan-3) were expressed in the tooth germs in various patterns and especially decorin, biglycan, syndecan-1 and syndecan-3 exhibited gene expressions overlapping with OASIS. Especially the expression pattern of decorin and syndecan-3 coincided temporally and spatially exactly with that of OASIS. They suggested that CREB3L1 gene might be related to proteoglycan expression.

Nikaido et al., (2002) demonstrated that the expression of the CREB3L1 mRNA was induced in response to the cryo-injury of the mouse cerebral cortex and found the OASIS-positive cells in the injured cortex similar to that of the glial fibrillary acidic protein (GFAP)-positive cells. The activation domain was found to be in the N-terminal region where acidic amino acids clustered and a possible repression domain, which had not been reported for other CREB/ATF family members, lay in the more C-terminal region. The authors proposed that OASIS protein positively regulated gene transcription in a subset of reactive astrocytes, and thereby influenced the reaction of injured central nervous system tissues. As gliosis is a characteristic response of astrocytes to inflammation and trauma of the (CNS) the authors suggested that OASIS protein may play a role in gliotic events. In various neurodegenerative diseases endoplasmic reticulum (ER) stress has been linked for neuronal death. Secretory and transmembrane proteins are correctly folded or processed in the ER, however, accumulation of unfolded proteins in ER lumen is observed in various stresses that alters the ER function. However, astrocytes in nervous system have ability to overcome ER stress and even proliferate under ischemic and hypoxic conditions that lead to ER stress. CREB3L1 is a novel ER stress transducer that regulates the signaling of the unfolded protein response specifically in astrocytes and contributes to resistance to ER stress (Saito et al., 2007).

Omori et al., (2002) isolated the human CREB3L1 gene and investigated the potential of this protein as a transcriptional activator. In concordance to mouse homolog human CREB3L1 also activates transcription through Box-B elements but not through the

somatostatin CRE. C-terminal hydrophobic transmembrane domain is a typical structural feature for the transcription factors activated by regulated intramembrane proteolysis. Human CREB3L1 contains a putative C-terminal hydrophobic transmembrane domain. Transcriptional activity was found to be significantly increased when the protein was truncated and it localized into nucleus from endoplasmic reticulum suggesting that transmembrane domain playing an important role in the regulation of transcription activation. . The protein was found to have molecular weight of 82k Da and 66 kDa in Western blot.

Following ER stress a fragment containing the cytoplasmic transcription factor domain is released by proteolysis. The cleavage is performed sequentially by site-1 and site-2 proteases (PS1 and PS2) and is triggered by translocation to the Golgi apparatus (Murakami et al., 2006).

NIH cDNA clone project has identified a clone expressing CREB3L1 in colon adenocarcinoma, indicating that this protein is involved in cancer apart from development and growth (Strausberg et al., 2002). In another study done by Novartis on breast cancer cell lines treated with lapatinib (0.0, 0.1g, 1.0g), the expression pattern did not vary on treatment for 2, 6 or 12 hours, however, lowest level of expression was found on MAD4638 cell lines, moderate in SKBR3 cell lines, and significant in BT474 cell lines (Straubery et al., 2002).

### 3.3. CYP17A1

Also called Steriod17-alpha-Monooxygenase, Cytochrome P450, Subfamily XVII, P450C17, S17AH, Steriod17-Hydroxylase/17,20-Lyase. Cytochrome P450 17alpha-hydroxylase (CYP17A1) is a single gene-encoding protein with two activities: 17alpha-hydroxylase and 17, 20-lyase. The two catalytic activities are differentially regulated in health and disease. 17-alpha-hydroxylase and 17,20-lyase activity functions allow the adrenal glands and gonads to synthesize both 17-alpha-hydroxylated glucocorticoids (via 17-alpha-hydroxylase activity) and sex steroids (via 17,20-lyase activity) (Chung et al., 1987; Kagimoto et al., 1988; Van Den Akker et al., 2002). Chung et al., (1987) isolated several human adrenal cDNAs corresponding to the P450C17 gene. The deduced 508-amino acid protein has a predicted molecular

mass of 57 kD. Picado-Leonard and Miller (1987) showed that the CYP17 and the CYP21B genes are very similar and presumably originated from a common ancestral gene. Matteson et al., (1986) concluded that the human genome has at least 2 P450C17 genes and that the adrenal P450C17 gene lies on chromosome 10.

Hanley et al., (2001) investigated the regulation of the human CYP17 gene by 2 orphan nuclear receptors, SF1 and DAX1 (dosage sensitive sex reversal DSS, adrenal hypoplasia congenita (AHC) critical region on the X chromosome, gene 1). In human embryos, SF1 and DAX1 are expressed throughout the developing adrenal cortex from its inception at 33 days postconception. In contrast, CYP17 expression, which commences between 41 and 44 days postconception, is limited to the fetal zone. The 5-prime-flanking region of the human CYP17 gene contains 3 functional SF1 elements that collectively mediate 25-fold or greater induction of promoter activity by SF1. In constructs containing all 3 functional SF1 elements, DAX1 inhibited this activation by at least 55%. In the presence of only 1 or 2 SF1 elements, DAX1 inhibition was lost even though SF1 transactivation persisted. The authors concluded that efficient repression of SF1-mediated activation of the human CYP17 gene by DAX1 requires multiple SF1 elements. Slominski et al., (1996) presented evidence that the CYP17, CYP11A1, CYP21A2, and ACTHR genes are expressed in skin. The authors suggested that expression of these genes may play a role in skin physiology and pathology and that cutaneous proopiomelanocortin activity may be autoregulated by a feedback mechanism involving glucocorticoids synthesized locally. Cytochrome P450c17 catalyzes both 17-alpha-hydroxylation and 17, 20-lyase conversion of 21-carbon steroids to 19-carbon precursors of sex steroids. In many species, the 17, 20-lyase activity of P450c17 for one pathway dominates, reflecting the preferred steroidogenic pathway of that species. To elucidate the pathway leading to C19 testosterone precursors in the human testis, Fluck et al., (2003) assayed the conversion of 17-alpha-hydroxypregnenolone to dehydroepiandrosterone (delta-5 17,20-lyase activity) and of 17-alpha-hydroxyprogesterone to androstenedione (delta-4 17,20-lyase activity) by human fetal testicular microsomes. Apparent Michaelis constant (Km) and maximum velocity (Vmax) values indicated 11-fold higher preference for the delta-5 pathway. They concluded that the majority of testosterone biosynthesis in the human testis proceeds through the conversion of pregnenolone to dehydroepiandrosterone via the delta-5 pathway.

Mammographic density is associated with increased breast cancer risk and is influenced by sex hormones. A T27C polymorphism (alleles A1 and A2, respectively) in the 5¢ promoter region of CYP17 may be associated with elevated sex hormone levels. In a cross-sectional study of 181 pre- and 173 postmenopausal women, Chi-Chen et al., (2004) examined the relationship of this polymorphism with mammographic density and other risk factors for breast cancer. CYP17 genotype was not associated with mammographic density levels before or after adjusting for risk factors for breast cancer. In premenopausal women, the A2 allele was associated with higher levels of dehydroepiandrosterone sulfate, and in postmenopausal women, with higher levels of total estradiol and lower levels of follicle stimulating hormone. Among premenopausal women, interactions were observed between CYP17 genotype and endogenous insulin levels as well as dietary variables associated with mammographic density. These findings suggest that the CYP17 A2 allele is associated with hormone levels, and interacts with insulin levels and diet to affect breast density levels and potentially breast cancer risk. (Chi-Chen et al., 2004)

In 2 patients with isolated 17, 20-lyase deficiency from a small village in Brazil, Geller et al., (1997) identified 2 different homozygous mutations in the CYP17A1 gene. In a study to identify genetic factors affecting the onset of menarche and natural menopause in healthy postmenopausal Japanese women, Gorai et al., (2003) found that ages at menarche in women with the CYP17 genotype A1/A2 (higher activity of CYP17) were significantly earlier than in those with A1/A1 (lower activity of CYP17). There were no significant differences in age at natural menopause and years of menstruation among each CYP17, CYP1A1 or COMT genotype. These results suggest that the estrogen-metabolizing CYP17 genotype influences age at menarche in healthy postmenopausal Japanese women. In 3 sisters with breast cancer diagnosed at ages 34, 38, and 42 years, respectively, Hopper et al.,(2005) identified a germline R239X mutation in the CYP17A1 gene.

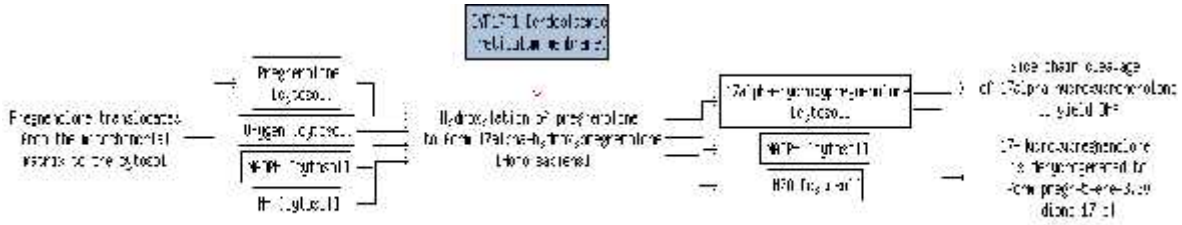


Fig 9 CYP17A1 mediated monooxygenase enzymatic pathway (Biason-Lauber et al., 2000)

17 alpha-Hydroxylase deficiency is characterized by defects in either or both the 17 alpha-hydroxylase/17, 20-lyase activities. Biason-Lauber et al., (2000) looked into the phosphorylation state of CYP 17A and found that cAMP stimulated phosphorylation of wild type and other mutants observed in clinical studies, however, for F417C, and R35L mutants cAMP did not stimulate phosphorylation. F417C has 17, 20-lyase deficiency. The F417C mutant protein lacks electron transfer potential and present finding provides for the first time a link between the electron transfer system and the phosphorylation state of the CYP17 enzyme in the control of 17, 20-lyase activity.

Ahlgren et al., (1992) for the first time, elucidated the molecular basis of the deficiency in a male pseudohermaphrodite with ambiguous external genitalia resulting from partial combined deficiency of both activities. They found that the patient is a compound heterozygote, carrying maternal allele having stop codon (TGA) at amino acid 239 arginine (CGA) in exon 4 and paternal allele that contains missense mutation encoding the substitution of proline (CCA) by threonine (ACA) at position 342 in exon 6. The stop codon occurs at N-terminal region of the heme binding pocket.

### 3.4. COX2

Also called Prostaglandin-Endoperoxide Synthase-2 (PGHS2), Glucocorticoid Regulated Inflammatory Prostaglandin Synthase, GRIPGHS. A major mechanism for regulation of prostaglandin synthesis occurs at the level of cyclooxygenase, also known as prostaglandin-endoperoxide synthase (PTGS). The first rate-limiting step in the conversion of arachidonic acid to prostaglandins is catalyzed by PTGS. Two isoforms of PTGS have been identified: PTGS1 (COX1) and a mitogen-inducible form, PTGS2. PTGS1 is involved in production of prostaglandins for cellular housekeeping functions, whereas PTGS2 is associated with biologic events such as

injury, inflammation, and proliferation (Hla and Neilson, 1992; Tazawa et al., 1994). In vitro translation of COX2 resulted in a 70-kD protein Hla and Neilson (1992). Kirschenbaum et al., (2000) concluded that PTGS2 is the predominant isoform expressed in the fetal male reproductive tract, and its expression may be regulated by androgens. Tsujii et al., (1998) used 2 in vitro model systems involving coculture of endothelial cells with colon carcinoma cells. Cells overexpressing COX2 produced prostaglandins and proangiogenic factors, and stimulated both endothelial migration and tube formation, whereas control cells had little activity. The effect was inhibited by antibodies to combinations of angiogenic factors, by NS-398 (a selective COX2 inhibitor), and by aspirin. NS-398 did not inhibit production of angiogenic factors or angiogenesis induced by COX2-negative cells. Tsujii et al., (1998) also found that COX2 can modulate production of angiogenic factors by colon cancer cells. Epithelial tumors may be regulated by COX enzyme products. To determine if COX2 expression and PGE2 synthesis are upregulated in cervical cancers, Sales et al., (2001) used real-time quantitative PCR and Western blot analysis to confirm COX2 RNA and protein expression in squamous cell carcinomas and adenocarcinomas. The authors concluded that COX2, EP2, and EP4 expression and PGE2 synthesis are upregulated in cervical cancer tissue and that PGE2 may regulate neoplastic cell function in cervical carcinoma in an autocrine/paracrine manner via the EP2/EP4 receptors. Salmenkivi et al., (2001) investigated the expression of COX2 in normal adrenal gland, in 92 primary pheochromocytomas, and in 6 metastases using immunohistochemistry and Northern blot and Western blot analyses. COX2 protein was expressed in the adrenal cortex, whereas the medulla was negative as detected by immunohistochemistry. Interestingly, all 8 malignant pheochromocytomas, regardless of the primary location of the tumor, showed moderate or strong COX2 immunoreactivity, whereas 75% of the 36 benign adrenal tumors showed no or only weak immunopositivity. These findings suggest that negative or weak COX2 expression in pheochromocytomas favors benign diagnosis.

Metastasis entails numerous biologic functions that collectively enable cancerous cells from a primary site to disseminate and overtake distant organs. Using genetic and pharmacologic approaches, Gupta et al., (2007) showed that the epidermal growth factor receptor ligand epiregulin, the cyclooxygenase COX2, and the matrix metalloproteinases MMP1 and MMP2, when expressed in human breast cancer cells,

collectively facilitate the assembly of new tumor blood vessels, the release of tumor cells into the circulation, and the breaching of lung capillaries by circulating tumor cells to seed pulmonary metastasis.

Mammalian species have two Cox isoforms; constitutively expressed cyclooxygenase-1 (Cox-1) and inducible cyclooxygenase-2 (Cox-2). Ishikawa and Herschman, (2006) cloned and characterized a second Cox-2 cDNA, Cox-2b, from the rainbow trout. This protein contained all the functionally important conserved amino acids for Cox enzyme activity that contained AU-rich elements (AREs) in the 3' untranslated region (3'UTR) indicating inducible Cox-2 mRNAs. Using a rainbow trout cell line to demonstrate that expression from both the originally reported Cox-2 (Cox-2a) and Cox-2b genes is inducible that observed differential induction responses to alternative inducers are for rainbow trout Cox-2a and Cox-2b. Both Cox-2a and Cox-2b proteins expressed in COS cells are enzymatically active. Thus the rainbow trout has two functional, inducible Cox-2 genes. The zebrafish also contains two Cox-2 genes. However, genome structure analysis suggests diversion of the Cox-2a gene between zebrafish and rainbow trout.

**3.5 NANOG**

Self renewable and pluripotency are two important characters of stem cell protein and NANOG has been found in regulating this important process. Constitutive expression of NANOG prevents differentiation of ESC to allow self renewal and which requires POU5F1 (OCT4) (Chambers et al., 2004). It has been proposed that actual stoichiometry between NANOG and OCT4 determines the fate of ESC. Expression of both genes is down-regulated during embryonic stem cell differentiation, with concomitant loss of pluripotency. (Hart et al., 2004). NANOG was also among the uniformly highly upregulated markers of the undifferentiated state recently identified by gene expression profiling of six different human ESC cell line. (Bhattacharya et al., 2004). NANOG is a new marker for testicular CIS (carcinoma in-situ) and germ cell tumors and the high level of NANOG along with OCT-4 are determinants of the stem cell-like pluripotency of the preinvasive CIS cell. Timing of NANOG down-regulation in fetal gonocytes suggests that NANOG may act as a regulatory factor up-stream to OCT-4 (Hoei-Hansen et al., 2005). Nanog is a transcription factor that plays key roles in the self-renewal and maintenance of pluripotency in human embryonic

stem (ES) cells. Among Nanog's 11 pseudogenes, NANOGP8 theoretically could be a retrogene, but was considered unlikely as it has not been identi.ed in any expressed sequence tags (ESTs). In this study, we found that NANOGP8 was expressed in several cancer cell lines and in all cancer tissues tested.

The expression of NANOGP8 in cancer cell lines and cancer tissues suggests that NANOGP8 may play important roles in tumorigenesis. This work not only has potential significance in stem cell and cancer research, but it also raises the possibility that some of the human pseudogenes may have regulatory functions (Zhang et al., 2006).

# CHAPTER-IV

## 4. MATERIALS AND METHODS

### 4.1 Material

The materials used in this thesis work are given in Appendix-I, II, and IV.

### 4.2 Methodology

For the detailed methodology it was followed as given in the Fig.

Expression profiling of proteins in tissue:

Clinical informations collection
↓
Filling of informed consent form and questionnaire
↓
Sample collection and transport
↓
Sample storage in liquid nitrogen
↓
Preparation of tissue lysate
↓
Determination of protein
↓
SDS PAGE electrophrosis
↓
Western bloting

Immunohistochemical analysis (ER/PR test)

1. Preparation of slides
2. Stainig procedure

Fig 10 Flow chart of methods

#### 4.2.1 Filling of informed consent form

The research was approved by the Ethical Review Board, Tribhuvan University Teaching Hospital, Kathmandu, Nepal. The informed consent form and questionnaire was asked to fill up by the relatives of patients having breast cancer and delivering

mothers, as suggested by Ethical Review Board Committee (given in Appendix-I and II).

### 4.2.2 Sample collection and transport

As soon as the breast operation was completed the cancerous tissue was given by the respective surgeon about 1gm of cancerous tissue was separated and put into a tube (overnight cooled at -20ºC). For placenta and umbilical cord, they were collected immediately after the laboring mothers have given birth to child.This was collected from gynanaecological department, TUTH. For ER, PR test breast cancer tissues were put in formalin.

### 4.2.3 Sample storage

The samples were well labeled (HBSC T1-T14 for breast cancer samples, HPL T1-T2 for human placenta and HUC T1-T2 for umbilical cord) and stored in a liquid nitrogen (-196ºC) until use. For immunohistochemistry analysis the tissue was stored in formalin.

### 4.2.4 Ripa buffer preparation

#### 4.2.4.1 Incomplete ripa buffer Preparation

Incomplete RIPA buffer was prepared mixing HCl, NaCl, EDTA, SDS and sodium deoxycholate needed for distruption of cell membrane (given in Appendix –IV).

#### 4.2.4.2. Complete RIPA buffer preparation

Incomplete RIPA was made complete by adding enzymes just before use (given in Appendix-IV).

### 4.5 Lysate Preparaion

1. About 0.3gm of tissue sample was weighted on a cooled glass slab.
2. The tissue was chopped into small pieces by a blade.
3. It was transferred into cooled homogenizer and required volume of complete RIPA buffer was added (0.057ml per gm of tissue).
4. The tissue was well grinded with homogenizer and after 15 minutes it was taken in a tube and centrifuged at 3,000 rpm for 3 minutes.
5. The supernatant was separated and stored at 4ºC until protein determination.

### 4.2.6 Determination of protein

The protein was determined by Lowery method as follow.

1. Six micro-centrifuge Eppendrof tubes (1.5 ml) were taken and labeled 0, 2, 4, 8, 12, and BSA (conc.1.5mg/ml) standard was pipetted into respective tubes with respective µl. For example in tube 2 the pipetted volume of BSA standard was 2µl.
2. The final volume of each tube was made up to 15 µl by adding the required volume of complete RIPA buffer.
3. Homoginized cancer tissue sample was diluted to 1:5, 1:20 and 1:50 by addition of complete RIPA buffer.
4. 15 µl of diluted sample was taken into clean micro-centrifuge tubes labeled T5, T20 and T50.
5. All the prepared tubes were vortexed and centrifuged for 30 seconds at 13,000 rpm.
6. In a 1.5ml microfuge tube, working solution of reagent was prepared by adding 20 µl of reagent S to 1ml of reagent A from BioRad.
7. 75 µl of reagent S and A mixture was added in all tubes and mixed well.
8. The tubes were centrifuged for 30 seconds at 13,000 rpm.
9. Then 600 µl of reagent B (BioRad) was added in each tube and vortexed immediately.
10. After 15 minutes absorbance of the standard and test samples were taken at 760nm.
11. The absorbance data of standard protein was entered onto protein determination program in excel to generate a calibration curve of the absorbance against concentration of standard. With the generated parameter

of linear constant a, absorbance y, standard concentration x the final concentration of test sample C was determined.

12. With the protein concentration data, the final concentration of the test sample was made 5 mg/ml for subsequent western blot analysis.

### 4.2.7 SDS PAGE electrophrosis and Western blotting

Antibodies to be tested, Strips prepared in PVDF, Mouse anti-goat IgG HRP (Jackson Immuno Research), TEMED, APS (ammonium per sulfate), 25%, Trizma base (Sigma), 0.1M Tween-20 (sigma), TBS, TBST (0.05% Tween in TBS), Blockng buffer (TBST with 3%w/v fat free milk.), Methanol, PVDF, Poncheu stain, Microcentrifuge tubes, pipettes.

**Procedure**

**Assembling for preparation of SDS-gel**

1. First of all the gel casting stand, casting frame and glass plates were cleaned and dried.
2. The holding stand for casting frames was put on a flat surface facing upward.
3. Glass plate with spacer is overlapped with another glass plate placing evenly at the bottom to prevent leakage of gel and to give space for loading sample at the top.
4. Both left end and right end of the overlapped plates were confirmed for proper overlapping and both the glass plates are even at both ends.
5. Evenly overlapped glass plates were then fitted into clamp unlocked frame.
6. After even leveling of glass plates and frame the clamps were locked.
7. The clamped frame and glass plates were then stuck on holding stand making sure the bottom of the clamped frame and glass plates are evenly attached to the rubber top of the holding stand.
8. The spring lever at the top of the holding stand was clamped to hold the clamped frame and glass evenly on the rubber top of holding stand.

9. Similarly another clamped frame with glass plates was fitted in another side of the holding stand.

**Gel casting**

10. For separation gel (resolving gel) solution 20 µl of 25% APS solution, 15 µl of TEMED was added in 8 ml of 12% gel solution (appendix IV) and was mixed properly by inverting for 3 times.
11. About 3.2 ml of final gel solution was pipetted smoothly between the plates. To avoid air bubble the gel was gently stirred with thin spatula.
12. Immediately the gel solution was overlaid with ethanol.
13. The acrylamide was allowed for polymerization (20-30 min).
14. After this the ethanol was drained inverting the gel and any remaining ethanol was absorbed by using clean filter paper.
15. Now stacking gel was prepared by adding 5 µl of 25% APS solution and 10 µl TEMED was added in 4 ml of 12% gel solution and was mixed properly by inverting three times. The final solution was then poured on top of casted separation gel till it reached the top of the short plate.
16. Appropriate comb was inserted between the spacer. The comb was inserted perpendicular to the gel.
17. The stacking gel was allowed to polymerize.
18. Gently the comb was removed after the stacking gel polymerized and the wells were rinsed thoroughly with running buffer.

**Electrophoretic separation of proteins**

19. The gel cassettes were removed from the casting stand and the frames were separated from glass plates containing gel.
20. The glass plates containing gel was placed into the slots in electrophoresis cassette with combed area at the top. Accordingly another glass plate containing gel was put in another slot.
21. The cassette containing gel plates was put into electrophoresis chamber with electrodes facing up ward.

22. Electrophoresis buffer (125 ml) was poured in the chamber filling the chamber containing cassettes and up to two third volumes of the outside chambers.

**Sample loading**

23. 1µl of loading buffer was loaded into each well,
24. For protein band size identification, 9 µl of loading buffer and 6 µl of protein marker (BioRad) was mixed properly and loaded in the right most well in both plates.
25. Equal volume of loading buffer was added into tissue lysate (5 mg protein/ml) and 5% V/V 2-ME was added to the lysate/buffer solution.
26. The solution was incubated for 5 minutes in boiling water.
27. Then it was immediately cooled in ice water to minimize proteolysis.
28. 14 µl of the sample solution was loaded in each well.

**Gel electrophroresis**

29. The chamber was covered with top containing power supplying socket for electrodes. Special care was given to make sure that the plus and minus socket was fixed to respective electrodes in cassette.
30. The gel tank was placed into a flat vessel containing ice slurry, submerging the tank about 2/3 of its height.
31. The top was connected to power pack (BioRad) for power supply.
32. Power pack was programmed for 200 volts and power was supplied for 77 minutes and run.

**Gel removal**

33. The power supply was turned off after it has stopped.
34. Carefully the inner chamber was lifted. The running buffer was discarded.
35. Gel cassette was removed from the chamber, and glass plates containing gel was removed from the cassette.
36. Gently the glass plates were separated from gel by gentle scrapping at the outer ends.

**Protein transfer**

37. The gel obtained after protein separation was covered by methanol soaked PVDF membrane filter at the front side of the gel. Special care was given not to allow air bubble between membrane filter and gel. The work was performed by adding transfer buffer during this step to avoid air bubble.

38. Other side of the gel was covered by filter paper and similar care was given to avoid air bubble.

39. Clamp containing foam was soaked in transfer buffer.

40. The sandwich of filter paper and gel is immersed in transfer buffer and placed between the clamps.

41. Avoiding air bubble the clamp is locked and is immediately transferred into chamber containing transfer buffer filled till the top of the chamber. Care was given to avoid air bubble.

42. The chamber was closed with its lead containing socket for power supply. Special attention was given to make sure that the plus and minus socket of the lead was fitted in respective electrode of the clamp.

43. The protein was transferred to membrane filter paper by supplying 100 volts for 60 minutes.

44. Protein separation pattern was confirmed by poncheu stain.

45. Transferred membrane was stored at 4$^{o}$C in a desiccator.

**Western blotting**

46. The transferred membrane filter was then cut into strips accordingly to the run well of the proteins, and labeled. The prepared strips were cut for the whole western assignment and labeled each strips with a strip number as assigned.

47. The test strips were submerged in methanol and incubated for 10 seconds. Then the strip was transferred in TBS (1X) solution and incubated for 5 minutes. Then it was transferred into TBS-T (1X) (Tween-20, 0.05%) for 15 minutes, and finally transferred into blocking buffer (3% skimmed milk in Tris Buffer Saline-Tween-20 for 40 minutes. Except for incubation in methanol, all other incubation was carried out on a rocking platform with moderate speed to allow the strip to evenly expose to the incubating solution.
48. The well of strip incubating plates was labeled as per the strips to be tested. Each well was filled with 2ml of blocking buffer. Proteins to be tested were separated by one empty well to prevent overflow of the antibody into other well designated to test another protein.
49. Then primary antibodies (specific antibody against target protein) were diluted according to the need.
50. The specific respective strips were transferred from blocking solution to the respective well of the strip incubating plate. The strips were completely submerged in the solution in the well.
51. The strips were incubated for 60 minutes on the rocking platform. The wells were gently tapped to submerge the strips.
52. After completion of incubation the strips were washed with TBST by incubating for 10 minutes, and were repeated for three times.
53. Secondary antibody (anti-goat IgG containing horse raddish peroxidase (HRPO) solution was prepared by diluting in the blocking buffer, at 1:25000 dilutions.
54. The well of incubating plate was filled with 2 ml of secondary antibody solution, and the strips were incubated in secondary antibody for 1 hour in rocking platform.
55. The strips were washed with washing buffer as described above.

**Development**

56. Solution A and Solution B were mixed in 1:40 ratio to prepare substrate of HRP for signal detection.

57. The strips were completely soaked by the substrate and were incubated for 3 minutes avoiding light.
58. Then the strips were taken and draining substrate was removed by gently soaking one end of the strip in filter paper.
59. The strips were gently fitted in X-ray plate.
60. Then the X-ray plate was exposed to X-ray light and the signal was trapped in Kodak X-ray film.
61. The exposure time of X-ray was for 1, 3 and 10 minutes to trap sufficient signal and to avoid unnecessary background.
62. After exposure the Kodak X-ray film was developed in developing solution submerging the film for 2 minutes.
63. Then the film was transferred into fixing solution and incubated for 1 minute.
64. Then the film was washed in water, and allowed to dry.
65. The corresponding protein marker band sizes were labeled on each strip X-ray image.
66. Corresponding band size of the dark signal generated by binding of specific antibody against the respective protein observed in the X-ray film was noted.

### 4.8 Immunohistochemical analysis of breast cancer tissue

**Materials**

PSB, citrate buffer, xylene, formalin, paraffin, streptavidin HRPO, haemtoxylin, hot air oven, waterbath, Microwave, alcohol(etanol), $H_2O_2$, Tris HCl Buffer, DAB, D.P.X.

**Procedure**

1. The tissue was fixed with 10% formalin. To achieve this, tissue should generally be small and thin (1×1×0.5cms), be fixed immediately as the fixation time should not exceed 24 hours.

2. The processing was completed using graded alcohols, xylene, substitutes and paraffin wax. During embedding, temperature greater than 60ºC must be avoided.

**Preparation of slides**

1. Section of 2 to 5 micron thick must be cut and well flattened on a waterbath.
2. These were mounted on glass slides coated with Poiy-L-Lysine or Gelatin – Chrome so that they adhere well and can withstand subsequent antigen retrieval procedure.

**Stainig procedure**

1. The slides were kept in hot air oven at 60ºC for one hour.
2. The slides were diped in xyline for 10 minutes twice.
3. The slides were dipped in alcohol (etanol) for 10 minutes twice.
4. The slides were dipped under burning tap water.
5. Then the slides were dipped under distilled water for 5 minutes.
6. The slides were removed from distilled water and added methanol and $H_2O_2$ in 9:1 ratio on each slides and incubated for 15minutes.
7. Slides were rinsed with distilled water.
8. The slides were put in Tris HCl buffer (Wash Buffer) for 5 minutes.
9. The slides were put in citrate buffer.
10. The slides were exposed in microwave in high power and low power. (5min high power and 25min. in low power).
11. After microwave procedure the jars were kept for cooling at room temperature for about 20min.
12. Then slides were put in wash buffer for 5 min.
13. Then the excess fluid was wiped and 1 to 2 drops blocking solution and incubated for 10 minutes.
14. The excess fluid was wiped and primary antibody was added and incubated for one hour at room temperature.
15. The slides were washed with wash buffer and incubated for 10 minutes.
16. Then excess buffer was wiped from the slides and streptavidin HRPO one to two drops and incubated for 30min.
17. After 30 min wash buffer was added and incubated for 5-10 minutes.

18. Excess buffer was removed from slides and 100 NI DAB working solution was added and incubated for 5-10 minutes at room temperature.
19. The slides were kept under running tap water for 5 minutes and haemtoxylin was added (1:10) for counter stain.
20. The slides were put in alcohol and xyline, 5 minutes each.
21. At last these slides were mounted with D.P.X. and observed under microscope at low power to high power.

# CHAPTER-V

## 5. RESULTS

### 5.1 Clinical analysis of collected samples

Within the study period from 15th July to 15th October 2007 total 14 patients came for breast cancer surgery at TUTH from different parts of Nepal. All the cancerous tissues were taken. Out of the total samples 57% were from Kathmandu valley, 28% from Terai and remaining 14% were from hilly region. The age range of patient was from 22-71 years. Out of the total patients two had menopause, 71% patients had children already while 14% do not have children. All patients were with tumors ranging from one to four, 46% with nodes and none of them had metastasis. Where 57% had vascular invasiveness and 71% had lymph invasiveness. According to the information nobody had family history of breast cancer. All the patients were noted under normal Nepalese food consumption and nobody was involved in regular exercise (Given in Appendix IV)

### Expression profile of proteins observed in western blotting

Western blot analysis of the protein using anti PP2A antibody contained distinct two bands one around 65kDa and one around 37kDa. 21.4% showed mild intensity, 35.7%showed strong intensity and 42.9%showed very strong intensity for around 65kDa with expression in whole tissues. Next band was seen around 37kDa and was found with mild intensity for 57.14%, 14.28%with strong intensity and 28.5%without expression. Both placenta samples were found high expressing of both bands and only single band was seen in umbilical cord.

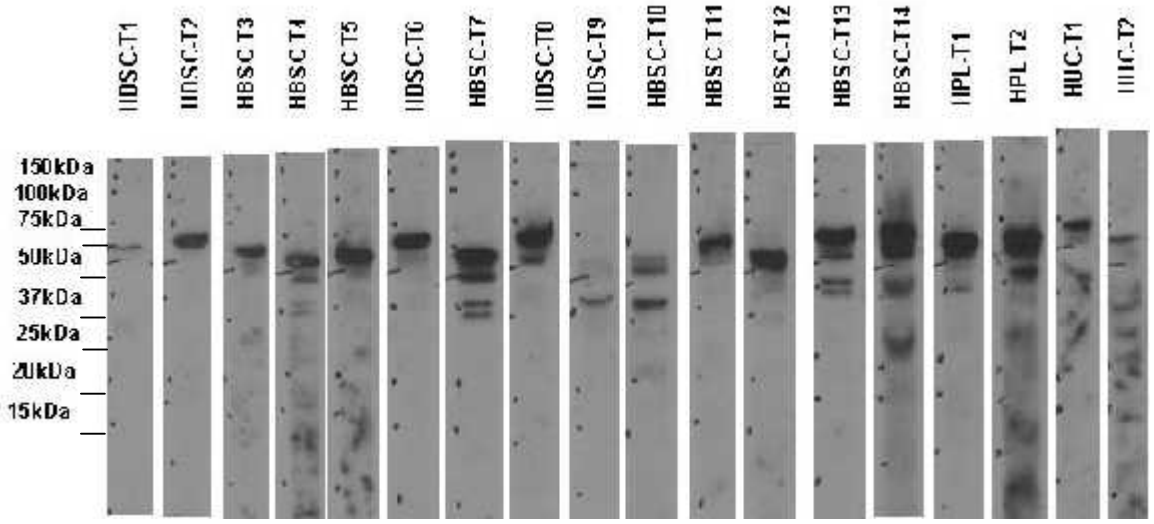


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Figure-12; Expression profile of PP2A in breast cancer, normal placenta and

Fig 11 Scanned figure of X-ray film after Western Bloting using anti PP2A antibody (HBSC- human breast cancer tissue, HPL- human placenta tissue and HUC- human umbilical cord)

**Table-2 Protein phosphatase 2A (around 65kDa)**

| Intensity | Frequency of the bands | Percentage |
|---|---|---|
| Mild | 3 | 21.4 |
| Strong | 5 | 35.7 |
| very strong | 6 | 42.9 |

**Table-3 Protein phosphatase 2A (around 37kDa)**

| Intensity | Frequency of the bands | Percentage |
|---|---|---|
| Mild | 8 | 57.4 |
| Strong | 2 | 14.28 |
| Negative | 4 | 28.57 |

Western blot analysis of CREB3L1, there were three bands corresponding to around 75kD, 55kD, 45kDa, 37kD and around 20kDa. Relative intensity of the band was observed (Table 2 and 3). In human placenta the bands observed were around 75kD, 55kD, 5kDa, 37kD and around 20kDa. In umbilical cord around 45kD and 55kDa band were seen.

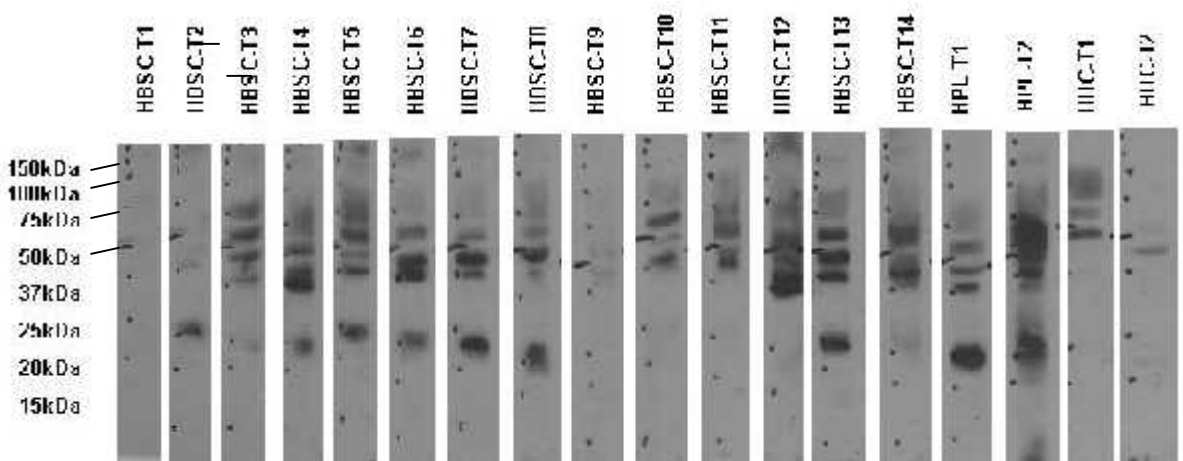

Fig 12 Expression profile of CREB3L1 in breast cancer, placenta and umbilical cord

**Table-4 cAMP element response binding protein 55 kDa**

Fig 12 Expression profile of CREB3L1 in breast cancer, placenta and umbilical cord

**Table-4 cAMP element response binding protein 55 kDa**

| Intensity | Frequency of the bands | Percentage |
|---|---|---|
| Mild | 6 | 42.9 |
| Strong | 2 | 14.3 |
| Negative | 6 | 42.9 |

**Table-5 cAMP element response binding protein for 38 kDa**

| Intensity | Frequency of the bands | Percentage |
|---|---|---|
| Mild | 6 | 42.9 |
| Strong | 5 | 35.7 |
| Negative | 3 | 21.4 |

**Table-6 cAMP element response binding protein for 48 kDa**

| Intensity | Frequency of the bands | Percentage |
|---|---|---|
| Mild | 5 | 35.7 |
| Strong | 4 | 28.6 |
| Very strong | 1 | 7.1 |
| Negative | 4 | 28.6 |

**Table-7 cAMP element response binding protein for 80 kDa**

| Intensity | Frequency of the bands | Percentage |
|---|---|---|
| Mild | 9 | 64.3 |
| Strong | 2 | 14.3 |
| Negative | 3 | 21.4 |

Western blot analysis for CYP 17A showed two distinct bands, bands around 75kDa showed 64.3% were with mild intensity and 35.7%were negative And for the band between 37kDa and 25kDa, 85.7% showed mild intensity and 14.3% were negative.Both placenta tissue matched with placenta but in case of umbilical caord only very mild expression was Observed for 75kDa and for the band between 37 and 25kDa only one sample showed positive and other was Completely negative. Except than these some bands were seen around 150kDa (Fig 13)

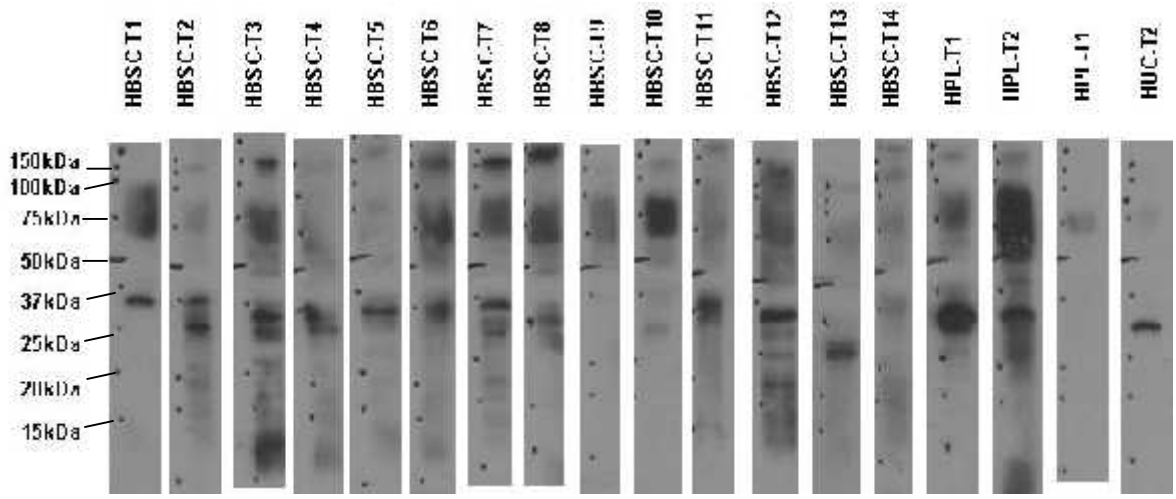


**Fig 13 Expression profile of CYP17A in breast cancer, placenta and umbilical cord tissue**

**Table-8 Cytochrome 17A for around 75kDa**

| Intensity | Frequency of the bands | Percentage |
|---|---|---|
| Mild | 9 | 64.3 |
| Negative | 5 | 35.7 |
| Total | 14 | 100 |

**Table-9 Cytochrome 17A for between 37kDa and 25kDa**

| Intensity | Frequency of the bands | Percentage |
|---|---|---|
| Mild | 12 | 85.7 |
| Negative | 2 | 14.3 |
| Total | 14 | 100 |

Western blot analysis for COX2 showed 50kDa band mild, strong, very strong and Negative intensity in 57%, 28.6%, 7.1% and 7.1% respectively. Both placenta showed high intensity for both bands where as in umbilical cord only one had high intensity.

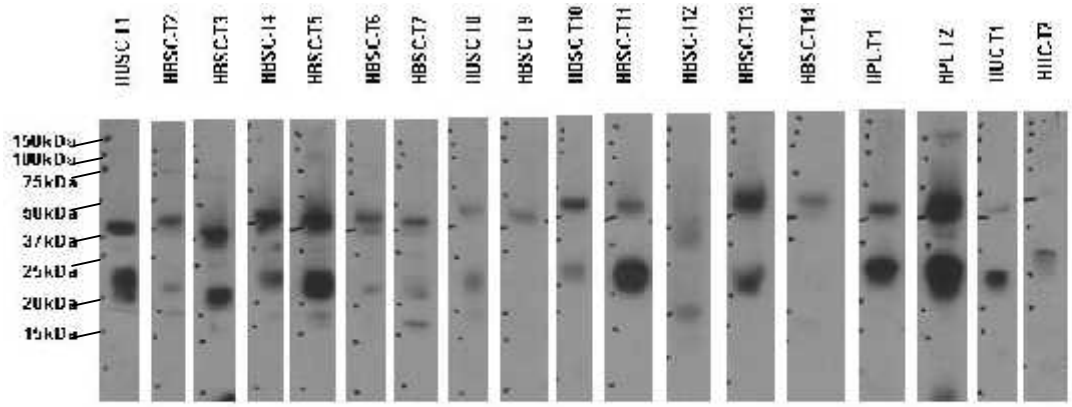


**Fig 14 Expression profile of COX2 in breast cancer, placenta and umbilical cord tissue**

| Intensity | Frequency of the bands | Percentage |
|---|---|---|
| Mild | 8 | 57.1 |
| Strong | 4 | 28.6 |
| very strong | 1 | 7.1 |
| Negative | 1 | 7.1 |

**Table-11 COX2 between 37and 25kDa**

| Intensity | Frequency of the bands | Percentage |
|---|---|---|

| | | |
|---|---|---|
| Mild | 6 | 42.9 |
| Strong | 3 | 21.4 |
| very strong | 3 | 21.4 |
| Negative | 2 | 14.3 |

Western blot analysis for NANOG showed three distinct bands as shown in figure tabulate in tables 13, 14 and 15.

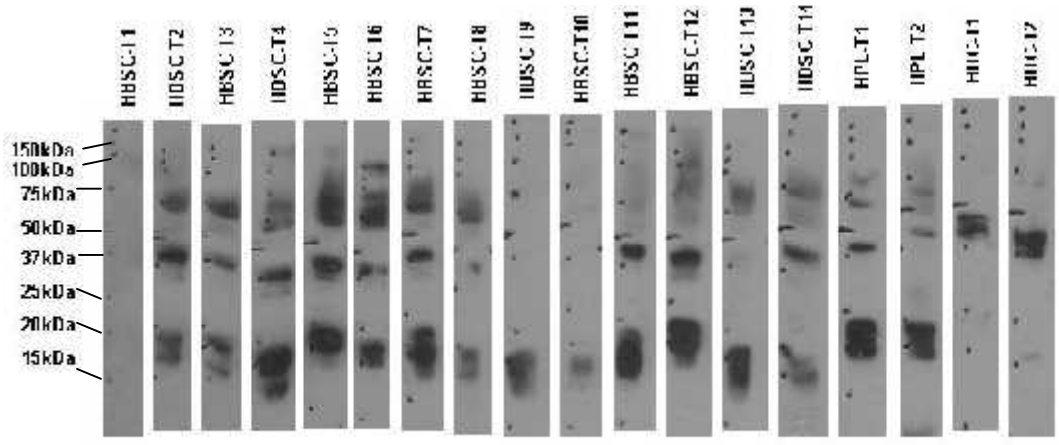


**Fig 15 Expression profile of NANOG in breast cancer, placenta and umbilical cord**

| Intensity | Frequency of the bands | Percentage |
|---|---|---|
| Mild | 8 | 57.1 |
| Strong | 4 | 28.6 |
| Negative | 2 | 14.3 |

**Table-13 NANOG for 75kDa**

| Intensity | Frequency of the bands | Percentage |
|---|---|---|
| Mild | 8 | 42.9 |
| Strong | 2 | 14.2 |
| Negative | 4 | 28.5 |

**Table-14 NANOG between 50 and 35kDa**

| Intensity | Frequency of the bands | Percentage |
|---|---|---|
| Mild | 5 | 35.7 |
| Strong | 4 | 28.6 |
| Negative | 5 | 35.7 |

# CHAPTER-VI

## 6.1 DISCISSION

PP2A is known to desphosphorylate SF1 and DAX1, and this dephosphorylated SF1 binds to promoter region of steroidogenic genes. Upon binding of CREB and SF1, steroidogenic genes are expressed. PP2A is reported to be expressed at low level in testis, ovary, thymus, brain, ovary in stem cell and carcinoma cell and highly expressed in testicular germ cell tumors, indicating its regulated expression in steroidogenic tissues and developmental tissues. Due to its expression in steroidogenic tissues and developmental tissues, correlation between steroids and breast cancer, and putative stem cell character of breast cancer tissue, PP2A was chosen for the study to see its expression profile in breast cancer.

In the present study, we have observed distinct bands around 70-60 kDa. The regulatory subunit of PP2A is found to migrate around 65 kDa in western blot analysis. In addition we have observed 40-37 kDa. The catalytic subunit is found to migrate around 36 kDa (Cohen et. al., 1989; Chowdhury et. al., 2005). Our result is in concordance to the finding of Chowdhury, Cohen.

Our result showed that the regulatory subunit to be highly expressed in all tissues but the catalytic subunit was seen only in about 71% of samples. As placenta and umbilical cord was used as a control group both placenta showed high intensity regulatory subunit band and weak catalytic subunit band where as only weak regulatory subunit was found in case of both umbilical cord. Thus it can be concluded that PP2A protein is highly expressed in breast cancer tissues as well as in placenta whereas it was weakly expressed in case of umbilical cord

Except in one sample (HBSC-T9) the regulatory subunit is expressed in all the breast cancer samples tested, however with varying degree of intensity. In placenta both regulatory and catalytic subunits are expressed, however, in umbilical cord distinct 65 kDa band is observed. In HBSC-T1 sample the regulatory subunit band is only observed and is relatively fainter than other samples. Although present study can not explain the reason why this band is fainter than others, but it could be possible that there might be some mutation or polymorphism in the promoter region of PP2A that

down regulates the expression of this protein in the sample. In addition, sample HBSC-T9 has distinct catalytic domain but has very faint signal for regulatory subunit. Although we can not explain through other literature search for this, but possibility could be that the exon intron junction for the regulatory subunit might have mutated so that there is mRNA transcribed however, the exon might have lost the exonic charater and the mRNA is not translated into protein, and only catalytic subunit is translated in protein.

In three of the breast cancer samples bands around 38 and 36 are expressed. Although we have not found literature to explain these two bands, but this could be possible splice variants of catalytic subunit. In five breast cancer samples additional band of 50 kDa is observed. Literature review revealed around 55 kDa splice variant, indicating that these patients might have carried splicing variants of regulatory subunit. Three out of five samples showing band around 50 kDa have bands doublet around 38 and 36 kDa, indicating that these patients might have heterozygous to splicing variants for both catalytic and regulatory subunit. However, this has to be verified through mRNA analysis.

As observed in testicular carcinoma, relatively high expression of PP2A in breast cancer indicates that this protein may implicate in carcinogenesis. Suziki et al., (2006) had reported that many breast cancer cell lines don't express PP2A, but E-cadherin expression is higher than normal breast tissues. However, the E-cadherin is endocytosed indicating that the cells have lost their potential for adhering and promoting cell proliferation resulting in metastasis of the cancer. Our present study did not find any tumor metastasis, but had higher expression of PP2A, indicating that E-cadherin endocytosis might have been prevented and allowed detection of PP2A. In addition, compared to umbilical cord, the expression level was found to be higher in breast cancer tissues, and placenta indicating that this protein might have role in the upregulation of steroid biosynthesis as placenta has higher level of hormone and breast cancer has been also reported to have higher steroid hormone biosynthesis.

The predicted band size of CREB3L1 is 57.0057 kDa, however, it is observed in three different band sizes, 80 kDa, 55 kDa, and 50 kDa, in endoplasmic reticulum stress (Saito et al., 2007). Yoshihiro et al., (2005) found this polypeptide with two different

molecular weight of 82kDa and 66kDa and suggested that the transmembrane domain plays an important role in the transcriptional activation by CREB3L1. CREB3L1 protein is ubiquitously expressed with high level in pancreas and prostrate. It is expressed relatively at lower level in brain (www.srs.ebi.ac.uk).

In the present study, we found CREB3L1 to be expressed in eleven of fourteen tumors tested. HBST-T1 had no expression; HBSC-T2 had very faint around 48 kDa and faint around 38 kDa and distinct band around 20 kDa; HBSC-T9 had very faint 55 kDa and 48 kDa. In predominantly CREB3L1 expressing sample, HBSC-T3 had bands around 80-75 kDa, 60-55 kDa, 48 kDa and faint around 38 kDa. HBSC-T4 had bands around 75 kDa, 55 kDa, 48 kDa and strong 38 kDa and around 20 kDa. HBSC-T5 had bands around 80-70 kDa, strong 55 kDa, 48 kDa, and 20 kDa. The bands around 80 kDa, 55 kDa, and 48 kDa can be explained as observed in ER stress. The additional bands around 38 kDa and 20 kDa could not be explained from present literature review, but similar 38 kDa band and strong 20 kDa bands in both placenta samples indicate that these bands could be due to the splicing variants, however, it has to be studied on mRNA level. This can be further explained that in HBSC-T13 which has faint 80 kDa band, but strong 55 kDa, 48 kDa, 38 kDa, and 20 kDa, and HBSC-T6 and HBSC-T7 that has no 80 kDa band but 55 kDa, 48 kDa, 38 kDa and 20 kDa. The overall pattern indicates that in some tumors CREB3L1 is not expressed, and in some 80 kDa protein is lost. In most cases 38 kDa and 20 kDa bands are observed indicating that in mammary tumor CREB3L1 is expressed and has splicing variant proteins of 38 kDa and 20 kDa. In umbilical cord, the band around the protein is expressed at 80 kDa, 60 kDa and 50-48 kDa in one sample indicating that our present antibody recognizes CREB3L1 as reported by (Saito et. al. 2006) and further explains that it can recognize splicing variants of 38 kDa and 20 kDa. In another sample of umbilical cord, the 80 kDa band is not observed indicating that the expression of protein corresponding to 80 kDa expression is down regulated in some individuals, as observed in some breast cancer samples, too. Although explanation for regulation of the expression of 80 kDa is not clear but functional analysis with promoter construct might explain the mechanism.

As this protein is expressed to maintain unfolded protein level in astrocites in nervous system and allow the cells to proliferate, present data indicate that the breast cancer

cells express CREB3L1 for its cell proliferation; however, this has to be confirmed through functional studies. In addition, present results indicate that 55 kDa, 48 kDa, and 38 kDa protein might play role in cell proliferation as it is observed in all positive samples and this has to be studied further. This is the first report of expression of CREB3L1 in most of the breast cancer tissues tested, and possible splicing variant.

The calculated molecular weight of CYP 17A protein is 57.371kDa, and we have observed band around 70-60 kDa in most of the samples tested. This is in concordance to the predicted molecular weight. However, in 9 samples faint band around 75 kDa has been observed. In Rhesus macaque CYP17A has shown band around 76.81 kDa has been observed. Our literature search did not find any explanation for human CYP17A showing band around 75 kDa. We assume that due to glycosylation and other post translational modification the band around 75 kDa has been observed as in Rhesus macaque.

In addition, we have observed band around 37 kDa in twelve samples. Literature search revealed that additional clone of CYP17A has 100 amino acids in N-terminus has been deleted (Yanase et al., 1990). Upon calculation of predicted molecular weight of the 100 amino acid deleted protein was around 46 kDa. Down stream to methionine in this 100 amino acid deleted protein, there is additional methionine. Upon calculation of predicted molecular weight from this downstream methionine, the protein size came around 42 kDa and antibody recognition amino acid sequence lies into protein. Taking into consideration of these observations, we assume that 37 kDa band might have been of either 100 amino acids deleted protein that has a splicing variant in downstream of antibody recognition sequence, or possible could be the 42 kDa protein that is transcribed from the methionine downstream of 100 amino acids deleted transcript.

Furthermore, we have observed 28 kDa in most of the tissues, and in placenta. In some cases stop codon (TGA) has been inserted in arginine at 239 amino acid of the protein (Ahlgren et al., 1992). Upon calculation of the 239 amino acids containg protein, the molecular weight was found to be around 27 kDa (Figure 13). We assume that the subjects showing band size around 28 kDa carries mutation at amino acid 239

and has stop codon at that amino acid. However, these findings have to be further validated by genetic profiling and sequencing of the mRNA transcribed.

CYP17A requires NADPH-P450 reductase, cytochrome b5 and phosphorylation of Serine/Threonine by a cAMp dependent kinase for 17,20 lyase activity. We have observed good correlation between PP2A and CYP17A1 expression in our study. This indicates that CYP17A1 is regulated by cAMP dependent pathway. In a study to identify genetic factors affecting the onset of menarche and natural menopause in healthy postmenopausal Japanese women, Gorai et al. (2003) found that ages at menarche in women with the CYP17 genotype A1/A2 (higher activity of CYP17) were significantly earlier than in those with A1/A1 (lower activity of CYP17). There were no significant differences in age at natural menopause and years of menstruation among each CYP17 genotype. In present study we did not find any differences between menarche age, breast cancer onset and expression profiling of CYP17A.

MWELVALLLL TLAYLFWPKR RCPGAKYPKS LLSLPLVGSL FLPRHGHMH NNFFKLQKKY GPIYSVRMGT KTTVIVGHHQ LAKEVLIKKG KDFSGRPQMA TLDIASNNRK GIAFADSGAH WQLHRRLAMA TFALFKDGDQ KLEKIICQEI STLCDMLATH NGQSIDISFP VFVAVTNVIS LICFNTSYKN GDPELNVIQN YNEGIIDNLS KDSLVDLVPW LKIFPNKTLE KLKSHVKIR

Theoretical pI/Mw: 9.65 / 27042.79 (Ahlgren et al., 1992).

COX2 is widely expressed protein in alimentary system (oesophagus, pharynx), male reproductive system (prostrate, seminal vesicle, ejaculatory duct), female reproductive system (cervix, uterus), hematopoietic system (bone marrow, monocytes). It is located intracellularly in cytoplasma, and microsomal membrane (European Bioinformatics, International Protein Index; www.srs.ebi.ac.uk). COX-2 is found to be implicated in colorectal cancer, gastric cancer, non small cell lung cancer, cholangiocarcinoma, uterine carcinoma, head and neck squamous cell cancer, ovarian cancer, pancreatic cancer, other malignant diseases, neuromuscular disorder etc. Furthermore, it is also found to be strongly expressed in breast cancer tissues (European Bioinformatics, International Protein Index).

Expression of mammalian Cox-2 genes is induced by wide variety of signals, including growth factors, cytokines, endotoxin, and neuronal depolarization, as well as in many pathophysiological conditions such as neuronal degeneration, inflammation and cancer [Herschman, 2004]. In the present study we have found COX2 to be expressed in breast cancer. We observed 55 kDa, 35 kDa and 25 kDa bands in our western results. Salhab et al. (2007) found COX-2/CK19 of both benign and malignant tissues were positively correlated with aromatase/CK19 transcript levels and concluded that COX-2 is an upregulator of aromatase in breast tissues. COX-2 is found to play a key role in tumorigenesis through stimulating epithelial cell proliferation, inhibiting apoptosis, stimulating angiogenesis, enhancing cell invasiveness, mediating immune suppression, and by increasing the production of mutagens. Studies with several epithelial cancers involving different organ sites, e.g., breast, prostate, bladder, lung, and pancreas, suggest that COX-2 plays an important role in the pathogenesis of these cancers. Studies from mouse models of mammary tumorigenesis and from human breast cancer cell lines provide evidence that COX-2 over expression plays an important role in the pathogenesis of malignant breast cancer in humans (Singh et. al., 2002). Detection of COX-2 in breast cancer tissue in present study is in concordance to the above reports.

Predicted molecular size of COX-2 is found to be around 68.99 kDa. In one of the studies, five heterozygous mutation (1missense/non sense, 1 splicing, 3 regulatory) was found in COX-2 gene. Two of them were associated with diabetes mellitus type 2, one with bladder cancer risk, one with increased risk of colorectal cancer and one with decreased risk of colorectal cancer (Tsujii et al., 1998). We have observed around 55 kDa in our samples. Taking into consideration splicing variant of COX-2, we assume that our 55 kDa band size is the splicing variant. However, this has to be validated through genetic study. In our study we have found high expression in placenta, also, and this is in concordance to the report that COX-2 is highly expressed in placenta (NCBI/OMIM).

Database searches suggest three cyclooxygenase genes are present in many fish species. Ishikawa and Harvery (2007) identified a second zebrafish Cox-2 orthologue, Cox-2b, and characterized this gene as a second inducible and functional Cox-2 homologue in the zebrafish. This was the first report of the expression of two distinct

inducible and functional Cox-2 isoforms in any species. Database searching suggests that some fish species have two potential Cox-1 genes, while other fish species have two Cox-2 genes, suggesting that duplication of alternative chromosomal regions during teleost evolution has resulted in differential retention of cyclooxygenase genes. Furthermore, two Cox-2 proteins have been observed in rainbow trout (Ishikawa et al., 2007). The bands observed around 35 kDa and 25 kDa could be alternatively induced COX-2 protein. This is the first report in human for alternatively induced COX-2 gene, however; this has to be validated through genetic study.

NANOG is found to be expressed in testis, ovary and thymus at lower level, but is expressed in embryonic stem cells and carcinoma cells and is highly expressed in testicular germ tumors (EBE/IPI). In the present study we looked into expression of NANOG to clarify the pleopotency of breast cancer cells. Calculated molecular weight of NANOG is found to be 34.620kDa. We observed expression of NANOG in present study. The observed band sizes were around 75 kDa, 45-40 kDa, and 20 kDa. Literature review did not reveal any reports for 75 kDa. As observed in CREB3L1 band corresponding to 80 kDa, 55 kDa and 50 kDa for predicted molecular weight of 57.0057 kDa for CREB3L1 (Saito et. al., 2007), the 75 kDa band size observed for NANOG could be due to similar fact for CREB3L1. The possible explanation could be that the protein is in dimer form, or glycosylation, phosphorylation and other post translational modification could have increased the band size.

NANOG has several isoforms and pseudogene. NANOG2 (NANOGP1) has molecular weight of 26.88kDa. Band size observed around 45-40 kDa is in close proximity of NANOG proteins band size of 34.62 kDa. Furthermore, stem cell associated transcript 8 is reported to have molecular weight 40.674 kDa. This protein is homologous to NANOG. This clearly supports that expression of NANOG is found in breast cancer tissue. In addition, the band observed around 20 kDa could be explained because development pluripotency associated protein 3 is reported to have molecular weight of 17.85 kDa. This protein shares homology with NANOG. NANOG has been reported to be expressed in breast cancer (Sell, 2007) at RNA level using real time PCR. Although real time PCR can detect RNA at low level, but it can not explain the size of the protein as only small region of RNA is amplified. We for the first time report that NANOG protein is expressed in breast cancer tissue at

protein level and the corresponding band sizes should be clarified through sequencing of mRNA

## 6.2 CONCLUSION

In the present study we investigated expression of CYP17A, PP2A, CREB3L1, COX-2, and NANOG in breast cancer tissue for identification of putative predicted biomarker for breast cancer susceptibility. The central hypothesis of present study was that steroid biosynthesis upregulation initiates tumorogenesis, proteins involved in stimulating epithelial cell proliferation, inhibiting apoptosis, stimulating angiogenesis, enhancing cell invasiveness, mediating immune suppression enhances cancer growth pleopotency of tumor cells promote rapid cell proliferation and tumor growth.

In one of the studies it was found that the increase in TAAAA short tandem repeats at promoter region of CYP11A shows high risk for breast cancer susceptibility, and homozygous to 8 short tandem repeat had around 2.94 times risk. In personal communication, it was learnt that 8 short tandem repeat had high expression of protein and was regulated by cAMP. We looked into expression of CYP17A as this is the enzyme that converts pregnolone to specific hormones through consecutive enzymatic activities of enzymes involved in steroid biosynthesis. We observed expression of CYP17A. We found expression of CYP17A around 70 kDa. Furthermore, we found 28 kDa mutant form which is generated be insertion of stop codon TGA in 239 arginine. This is the first report for 28 kDa CYP17A in breast cancer. All of our brest cancer tissues carried this allele. The N-terminus of CYP17A is responsible for binding of NADPH-P450 reductase for electron transfer to heme pocket, we can not explain from present study the implication of this truncated protein in breast cancer. In addition, we have observed 40 kDa variant of CYP17A. Literature review had revealed additional clone of around 1.3 kb which is shorter than normal around 1.7 kb transcripts and has lost around 100 amino acids in N-terminus. Molecular weight calculation predicted the molecular size of this short transcript to be around 46 kDa. Downstream 30 amino acids from putative protein synthesis initiation methionine (98 amino acid of normal transcript) of this shorter transcript there is additional methionine (128 amino acid of normal transcript). Assuming that the

protein synthesis could have been initiated from 128, methionine, molecular weight calculation predicted the size of the protein to be around 42 kDa. We assume that the band corresponding around 40 kDa could be alternatively spliced protein that is transcribed from 128, methionine. This is the first report for additional transcript of CYP17A. Loss of N-terminus aminoacids results in loss of NADPH-P450 binding domain making the protein incapable of electron transfer for monooxygenase activity, expression of this alternatively spliced protein in breast cancer could not be explained from present study.

Steroid biosynthesis genes (steroidogenic genes) are expressed after cAMP response, that is regulated by PKA mediated signaling cascade. PP2A is responsible for dephosphorylatiohn of SF-1 which then enters nucleus and binds to SF-1 binding domain at the promoter region of steroidogenic genes and upregulates expression of these genes after binding of CREB in its binding domain in promoter region. We looked into expression of PP2A. We observed expression of regulatory subunit in all the samples, however, catalytic subunit to be expressed only in 71% of the samples. This indicates that the role of PP2A in breast cancer is not only dephosphorylation of proteins down stream of PKA cascade, but additional role of regulation of signal cascade.

ER stress induced expression of CREB3L1, and being in ATF family we thought that this protein could be expressed in breast cancer to promote expression of steroidogenic genes contrary to the CREB that regulates expression of steroidogenic genes in hormone synthesis tissues such as ovary and adrenal. We observed expression of CREB3L1. In addition, we observed splicing variants of around 38 kDa and 20 kDa. One of the roles of this protein could be that it binds to N-terminus region of CYP17A and promotes transcription of 42 kDa variant. In addition, it might regulate expression of COX-2 variant (COX-2b) as observed expression in our study.

COX-2 expression and CYP19 (aromatase) expression has been found to be correlated. Aromatase is involved in biosynthesis of oestrogen, and oestrogen has been found to be correlated with breast cancer. We observed high expression of COX-2 in breast cancer tissue. However, this was shorter than predicted molecular weight indicating that in breast cancer splicing variant of COX-2 is expressed. In addition,

we found expression of COX-2 variants, and literature review revealed functional Cox-2 variants in rainbow trout and zebrafish that is transcribed from same gene. Taking into consideration of these Cox-2 variants in fishes, we assume that our shorter proteins of 35 kDa and 25 kDa are COX-2 variant expressed from same gene. One of the explanation of expression of variants of COX-2 could be that CREB3L1 might bind to 5'-region of COX-2 mRNA and initiates expression of variant forms. This is the first report that in breast cancer splicing variant of COX-2 is expressed. In addition, this is the first report indicating presence of COX-2 variants in human.

In order to clarify whether breast cancer tissue has pleopotency, we looked into expression of ES protein NANOG. We found expression of NANOG in breast cancer tissue. In addition, we found additional variant of 20 kDa that corresponds to the molecular size of Developmental pluripotency associated protein 3, which is also homeobox domain protein homolog of NANOG. This is in concordance to other reports that has reported expression of NANOG in breast cancer.

# CHAPTER-VII

## 7 SUMMARY AND RECOMMENDATIONS

## 7.1 SUMMARY

1. Fourteen breast cancer tissue samples were collected from Surgery Department, two umbilical cord and two placenta were collected from Department of Gynaecology, Trivuwan University, Teaching Hospital, Maharajgung, Kathmandu.

2. All the samples were made lysate and protein was separated by SDS PAGE electrophrosis and the proteins of interest (CYP17A, PP2A, COX2, CREB3L1 and NANOG. Of them two proteins were involved in metabolism of steroid, two were involved in regulation pathway and one was stem cell protein) were detected by Western Bloting.

3. Clinical data and history of patient was obtained from TUTH, however ER/PR result was incomplete.

4. The expression level and molecular weight in different tissues were obtained from different websites (www.srs.ebi.ac.uk, www.expasy.com, www.hprd.com , Pubmed/OMIM etc) and literature review.

5. In most of the tissue the proteins expressed well as the proteins selected were directly involved in steroid biosynthesis. With the over production of steroid hormones, these steroid metabolism and regulatory proteins were also found to be expressed high.

6. Among the selected proteins for CYP 17A, CREB3L1 and COX-2a no any literature was found in breast cancer studied in expression by Western bloting. These proteins were found to be well expressed in our result with some defined and some undefined molecular weight. We think these may be putative breast cancer biomarker and should be studied in genomic level.

## 7.2 RECOMMENDATION

1. Our present study is limited with the sample numbers we could not correlate with the protein expression profile and breast cancer status, so it is customary to increase the sample size to validate any predictive biomarker for breast cancer susceptibility. Furthermore, our finding of splicing variants, and transcription variants of proteins is limited with RNA.
2. DNA analysis to further validate the finding, it prudent to do analysis at genomic level to strengthening our findings.
3. Present study was conducted only to see the expression profile of different genes, we could not elucidate the role of some of the proteins, or their splicing variants due to the lack of functional studies. In order to elucidate the mechanism of these proteins and their splicing variants cell culture based functional study is important.

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UCSF News Office 3333 California Street Suite 103, 29th November 2005

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