Establishment of the human placenta is essential for subsequent development of the embryo. Anchoring of the fetal placenta to the uterine wall requires the differentiation of a highly specialized population of placental epithelial cells, the fetal cytotrophoblasts (Pijnenborg, 1990). Cytotrophoblast stem cells of first-trimester chorionic villi form a continuous epithelial layer attached to a basement membrane. Differentiation along either of the two alternative pathways begins when cytotrophoblasts detach from this basement membrane. In one pathway, cytotrophoblasts fuse to form the syncytiotrophoblast layer that covers the chorionic villi (floating villi). In the second pathway, cytotrophoblasts aggregate to form columns and then invade the uterine wall and its associated arterioles, thereby breaching the maternal circulation. This process peaks during early pregnancy, rapidly declining during the second trimester (Fisher et al., 1989; Yeh and Kurman, 1989; Pijnenborg, 1990). Thus trophoblast invasion is an interesting example of extremely aggressive, but closely regulated, invasive activity of a normal cell. Uncontrolled cellular proliferation and invasiveness are the two essential features of malignant phenotype and the transition of a normal cell to a malignant cell usually involves a loss or derangement in one or more of the control mechanisms.

Hydatidiform mole is considered to be a hyperproliferative condition of the normal placenta. Hydatidiform mole, even though not considered malignant shows various characteristics of malignancy including invasiveness which is frequently fatal (Lurain and Brewer, 1982; Song et al., 1998). Altered expression of different
genes might be a factor leading to this tumourigenic condition as in the case of other cancers. Altered forms of genes or their protein products are documented to be potential prognostic markers in different types of cancers. The aim of this study was to look into the progression of the disease at the levels of aberrant expression and co-expression of the oncogenes, tumour suppressor genes, proliferation markers, apoptosis related genes, metastasis related genes and viral proteins in CHM which could be instrumental in increased proliferation, invasion and persistence.

The world-wide incidence of GTD varies from 0.5 to 12 per thousand births (Fig. 1). India has a high incidence of this disease (Pai, 1967), Kerala reporting a hospital incidence as high as 12/1000 deliveries (Molykutty et al., 1993). The incidence data of this study was also in accordance with our previous reports (Prabha et al., 1995), however, the actual incidence might be lower as the SAT Hospital is a referral hospital for this disease. Our analysis showed that about 70% of the patients were in the age group 21-30 (years) (Fig. 3). Regarding the obstetric history, the multiparae and among them those with previous abortion had an increased risk of the disease. Recurrent vesicular mole was of rare occurrence.

In the present study, majority of the molar placentae were of mid gestation and the molar placentae presenting in the late gestational ages were comparatively few. Moreover, we also noticed a significant association between gestational age of presentation of the disease and prognosis. We observed that persistence of the
disease after evacuation was comparatively less in >12 week gestational age group (Fig. 4). Gestational age emerged out as an independent prognostic indicator of persistence of the disease after evacuation in multivariate analysis also. (Table-56).

**ONCOGENES**

Mammalian embryo participates in a complex dialogue with the maternal physiology. The language of this dialogue is growth factor signalling. The trophoblastic cells show the expression of a cascade of growth factors and receptors involved in their proliferation. Therefore, in the present study, the expression of the growth factors EGF and TGF-α, the growth factor receptors, EGFR and c-erbB-2 and signal transducers p21ras and c-myc were evaluated in CHM and normal placentae.

**EGF**

Cell proliferation mediated by EGF is a central event in normal as well as neoplastic tissue. EGF plays an important role during pre-implantation mammalian development (Harvey et al., 1995). EGF stimulates the differentiation process of the cytotrophoblasts to form the syncytium (Dakour et al., 1999). Increasing evidence indicates that EGF acts as an enhancer of trophoblast function to produce hCG (Cao et al., 1994).
In the present study also, EGF expression was detected in all the gestational ages in normal placentae suggesting its role in normal placental development. The expression was detected to be higher in the early stages of pregnancy linking its role to early proliferation and differentiation in normal placentae (Table-4, Plates 3A & B). Thus the presence of higher amounts of EGF appears to play an important role in proliferation and differentiation of trophoblasts. In parallel with our observations, Ladines Llave et al. (1991) and Bissonnette et al. (1992a) have also detected a phasic pattern of expression of EGF in normal placentae. Hofmann et al. (1991) have immunolocalized EGF to uterine epithelial cells, decidual cells as well as to cytrophloblasts and syncytiotrophoblasts. However, Bass et al. (1994) have failed to detect the presence of EGF in early trophoblast culture medium. In the present study, EGF was overexpressed in the extra-villous trophoblasts of the normal placentae also. It has been reported that the invasion of the "extravillous" subpopulation of normal placental trophoblast cells depends on mechanisms identical to those of invasive cancer cells (Yagel et al., 1988; Lala and Graham, 1990). Their invasiveness has been shown to be regulated by certain locally derived growth factors, growth factor binding proteins and other molecules in the extracellular matrix (Lala and Lysiak, 1995; Lala and Hamilton, 1996).

The EGF expression was seen to be higher in the molar placentae in all the gestational ages (Table-4 & Plate 3C). The significantly increased expression of EGF in molar placentae may provide a growth advantage to the molar tissue. The molar placentae of the second trimester also exhibited higher staining for EGF,
whereas none of the second trimester normal placentae were intensely positive (Fig. 5). The EGF expression also showed a significant increase with the aggressiveness of the disease (Plates 3E & F). Higher expression of EGF is of prognostic value in breast cancer (Messa et al., 1998). We also noticed a significantly higher expression in the chemotherapy group compared to the spontaneously and slowly regressing lesions (Table-5). A significantly higher expression in the invasive trophoblastic lesions was also seen (Table-6). Bass et al. (1994) have demonstrated that addition of EGF to first trimester cytotrophoblast cultures produced dramatic changes in morphology and a several fold increase in the invasive capacity. Previous studies from our group have also reported increased EGF expression in the molar tissue and choriocarcinoma (John et al., 1997a & b).

In the present study, using univariate analysis we observed that overexpression of EGF could be a significant predictor of prognosis and invasiveness of molar pregnancies. However, in multivariate analysis EGF expression did not show a significant relation with either persistence or invasiveness of the disease.

TGF-α

As in the case of EGF, TGF-α was expressed at a high intensity by the normal placentae in all the gestational ages (Table-7, Fig. 8 & Plate 4A). This points towards its role in normal placental development. Haining et al. (1991) have demonstrated the presence of TGF-α and EGF mRNA in the human endometrium and term decidua. Bissonnette et al. (1992a) and Filla et al. (1993) have also immunolocalized TGF-α in human trophoblast and decidua. It has been
suggested that these peptides may play a role in the epithelial regeneration after menstruation and the mitogenesis and growth of the trophoblasts. Lysiak et al. (1993, 1994) have reported the role of TGF-α in the maintenance of the growth activity of the trophoblasts.

TGF-α expression has been linked to the prognosis of different tumours (Murray et al., 1993; Wang et al., 1996, 1997; Messa et al., 1998). In the present study, a higher expression of TGF-α has been localised in the molar placentae also, suggesting its role in trophoblastic proliferation (Plate 4C). The expression was also significantly increased in the persisting disease group compared to the spontaneously regressing group (Table-8 & Plates 4E to F). We found significant difference in TGF-α expression with the persistence of the disease in multivariate analysis (Table-56), suggesting that TGF-α can be used as an independent prognostic indicator of persistence of the disease. However, the expression pattern did not show any relation with the invasive nature of the disease (Table-9). Therefore, TGF-α may be linked to trophoblast proliferation and persistence of the disease rather than invasion.

**EGFR**

A phasic expression of EGFR has been reported in normal placentae (Ladines Llave et al., 1991; Bissonnette et al., 1992a). In the studies of Jokhi et al. (1994) EGFR was immunohistochemically localised in the proliferative villous cytотrophoblasts but not by non-proliferative, invasive extra-villous cytотrophoblasts.
In the present study, EGFR expression was detected in all the gestational ages in the normal placentae (Table-10, Fig. 11 & Plate 5A). The presence of the growth factors, EGF, TGF-α and the receptor EGFR suggest autocrine mechanism of action of these proteins in normal placenta thereby exerting their mitogenic effect.

Fujita et al. (1991) have reported a decrease in EGFR and its mRNA levels in intrauterine growth-retarded and diabetes mellitus-complicated pregnancies suggesting a possible physiological action of EGF on adequate feto-placental growth and development in human pregnancy. Cao et al. (1995) have reported the presence of functional nuclear EGFR in human choriocarcinoma JEG-3 cells and normal human placentae. According to them, nuclear receptors have the capacity to transduce the signals from EGF and may mediate intracrine and paracrine actions of EGF in the regulation of trophoblast functions. Presence of an autocrine mechanism of EGF in choriocarcinoma cell proliferation has been detected by Nomura et al. (1996). Previous studies from our group have also reported increased EGFR expression in molar placentae (Balaram et al., 1997; John et al., 1997a, 1999). In the present study also, EGFR expression was higher in the molar placentae with increased EGF expression (Fig. 11), thereby suggesting autocrine mechanisms of action. In univariate analysis, we observed a significance for the expression of EGFR with persistence of the disease (Table-11) but it lacked significance in multivariate analysis.

We observed increased EGFR expression in the invasive lesions (Table-12 & Fig. 13). EGFR expression has been shown to be correlated with invasive phenotype in human prostate adenocarcinoma Du 145 cells both in vitro
and *in vivo* (Xie *et al.*, 1995; Prewett *et al.*, 1996). The significant increase of both EGF and EGFR in the invasive lesions point towards an autocrine mechanism active in the invasive lesions. However, they lacked significance in multivariate analysis.

**c-erbB-2**

As indicated above, EGFR was found to be of prognostic significance in CHM and there are also evidences of interaction (transmodulation) between EGFR and *c-erbB-2* product in other cancers (Goldman *et al.*, 1990; Wada *et al.*, 1990; Spivak-Kroizman *et al.*, 1992). In tumour cell lines expressing both receptors, treatment with EGF can result in phosphorylation of the *c-erbB-2* protein also. Therefore, we have also analysed the expression of *c-erbB-2* in these patients, to further investigate the prognostic significance of this protein and also its interaction with other proteins, especially EGFR as discussed later.

The *c-erbB-2* expression was localised to both the cytotrophoblasts and syncytiotrophoblasts of normal placentae (Table-13 & Plate 6A). We observed cytoplasmic as well as membraneous staining for *c-erbB-2*. The significance of cytoplasmic staining is unclear. De Potter *et al.* (1989) showed that this pattern was related to a 155 kD protein, which may be a degradation product or a precursor of the 185 kD *c-erbB-2* protein. This pattern is found frequently in other tumours and in some normal fetal and adult tissues (Press *et al.*, 1990), whereas
in breast cancer membrane staining has been regarded as specific for this oncprotein (Haugen et al., 1992; Coombs et al., 1993). The cytoplasmic localisation of this protein has been suggested to be due to disturbance in the transport of this protein to the cell membrane (Osako et al., 1998).

In the study of Jokhi et al. (1994) the expression of c-erbB-2 was seen in the invasive extravillous trophoblasts but not in the villous cytотrophoblasts. In their study, EGFR and c-erbB-2 were expressed in terminally differentiated placental bed giant cell and villous syncytiotrophoblast populations suggesting EGFR to play important role in trophoblastic proliferation whereas c-erbB-2 to be important in trophoblastic invasion and differentiation. We did not detect a significant phasic pattern of expression in the normal placentae for c-erbB-2. Bauer et al. (1997) have reported a relation between c-erbB-2 amplification and DNA content and karyotype.

In the present study, c-erbB-2 was also overexpressed in the molar placentae compared to normal placentae (Table-13 & Plates 6C to F). However, Cameron et al. (1994) have reported a negative staining for c-erbB-2 in early and term normal placentae, spontaneously regressing and persistent gestational trophoblastic disease as well as in choriocarcinoma. Of the 56 cases analysed by them, only one sample belonging to the persistent disease group showed moderate positivity for c-erbB-2. But in their study they used archival material and they themselves have suggested that this may be the possible reason for the negative
staining. Jokhi et al. (1994) have reported a lack of difference in either EGFR or c-erbB-2 expression by normal and malignant trophoblasts by flow cytometry. Fulop et al. (1998) have also detected increased c-erbB-2 staining in the molar placentae, similar to the results of the present study.

The expression of this protein showed no statistically significant difference in the invasive and non-invasive lesions (Table-15). Berchuck et al. (1991) have showed that the heavy staining of c-erbB-2 was associated with increased mortality from persistent or recurrent endometrial carcinoma as well as with the presence of metastatic disease, increasing histological grade and depth of myometrial invasion, although the two latter prognostic factors were not statistically significant. High level amplification of c-erbB-2 has been related to the progression and metastasis of cancer (King et al., 1990; Houston et al., 1999). In the present study, c-erbB-2 showed a significant increase in the persistent disease group compared to the spontaneously regressing group (Table-14 & Plate 6F), but in multivariate analysis it does not hold good promise as a marker of the grade or persistence of the disease. But the relations with ligands of the erb-B family and the receptor EGFR suggest synergistic role along with other proteins such as EGFR through a heterodimerisation mechanism as discussed later.

ras

The members of the ras oncogene family have received attention on account of the transduction of extra-cellular signals which affect cell proliferation,
differentiation and the regulation of cytoskeletal dynamics. Watari et al. (1996) indicated that H-ras gene may facilitate the vectorial transcellular fluid transport from maternal site to fetus, while K-ras gene is associated with some endocrine functions such as hCG production in trophoblasts. Kenton and Johnson (1994) have detected p21ras in the human term placental syncytiotrophoblast plasma membrane. We observed p21ras expression in the normal placentae throughout gestation (Table-16 & Fig. 17). The expression was more in the early gestational ages suggesting increased signal transduction mechanisms resulting in increased proliferation (Plate 7A).

Ras GTPase activating protein (Ras GAP), a major down-regulator of ras activity is present at high levels in placenta and non-invasive moles, while choriocarcinomas have shown a lack of expression of Ras-GAP (Stahle-Backdhal et al., 1995). Sasa et al. (1997) have demonstrated decreased expression of p100-GAP protein in comparison to p120-GAP in mole villi compared to normal chorionic villi of first trimester indicating its role in normal growth and differentiation of human trophoblasts of the first trimester. Ye et al. (1999) have discovered a novel ras-GAP-associated protein of 105 kD in both mature trophoblasts and differentiating choriocarcinoma cells linking its role to the differentiation state of the trophoblasts.

An increased expression of rasp21 was evident in the molar placentae in the present study also (Table-16 & Plates 7D to F). The expression was maximum in the third trimester (Fig. 17). Fulop et al. (1998) have reported a lack of
mutation for K-ras in complete moles and choriocarcinomas. We observed no relation of ras expression with the prognosis or invasiveness of the disease (Tables- 17 & 18).

c-myc

A diffuse nuclear staining for c-myc is reported in early cytotrophoblasts and both layers of the mid-gestation trophoblasts and undetected in full-term normal placenta (Roncalli et al., 1994). We observed c-myc positivity in all the gestational ages and the staining intensity was comparatively less in late gestation (Table-19, Plates 8A & B).

There are reports on the expression of c-myc in the cytotrophoblasts of early placentae and in the cytotrophoblasts and syncytiotrophoblasts of hydatidiform moles and choriocarcinomas (Sarkar et al., 1986; Yokoyama et al., 1988). Cheung et al. (1993a) have reported a very low expression of c-myc in both hydatidiform moles and normal placentae. According to them c-myc had no role in predicting the prognosis of molar pregnancies. We could find intense staining for c-myc in the chemotherapy group (Plate 8E) and in the invasive lesions, but c-myc expression showed no significance with the prognosis of the disease (Table-20). Fulop et al. (1998) have reported a significantly stronger staining for c-myc in the syncytiotrophoblastic layer of the normal placenta, complete mole and choriocarcinoma compared to the partial mole.
TUMOUR SUPPRESSOR GENES

Numerous mechanisms that underlie oncogenic transformation have been identified. They predominantly involve alterations in proliferation-related signal transduction, which lead to sustained cell growth without cell differentiation. Tumour suppressor proteins have been shown to be capable of abolishing such sustained growth by interfering with cell cycle progression, such interference having been considered to form a specific surveillance mechanism (Levine et al., 1994). Tumour suppressor genes may have a role in the control of trophoblast cell population expansion as trophoblast invasion occurs. The expression of the tumour suppressor proteins Rb, p53 and p21 were evaluated in the present study.

Rb

In neoplasia, nonfunctional mutant Rb, low Rb expression and maximally phosphorylated Rb have been described, indicating the potential mechanisms by which neoplastic cells can escape from cell cycle control in various types of tumours (Pavelic et al., 1996; Kaelin, 1997; Kornblau et al., 1998; Pande et al., 1998; Volm et al., 1998). During G1 phase hypophosphorylated Rb appears to be tightly associated with nuclear structures (Mittnacht and Weinberg, 1991; Templeton, 1991). Ordinarily, under-phosphorylated Rb, through its sequestration of transcription factor E2F1, inhibits the cell cycle. Phosphorylation of Rb leads to release of E2F1 and subsequent transcription of genes that are important in moving the cell from the G1-phase into the S-phase (Bartek et al., 1997; Connell-Crowley et al., 1997).
In the present study, we have detected an overexpression of the Rb protein in the complete moles (Table-22 & Plate 9B). The over-expression of Rb noted in CHM in this study, could not be related to mutant Rb or phosphorylation state, because the antibody to Rb could not distinguish between these proteins. Therefore, the mechanism behind overexpression in these tumours remains to be determined. The overexpression, may be due to the high proliferative rate of the tissue.

However, a significantly decreased expression of Rb was noticed in invasive cases of CHM suggesting a role for the normal Rb protein with the invasive nature of the disease (Table-24 & Plate 9F). Wright et al. (1995) have confirmed the association of the absence of Rb protein with invasive growth and high-grade of different tumours. Similar results were obtained by Dosaka-Akita et al. (1996) in non-small cell lung cancer. The Rb protein status was not correlated with TNM, survival or prognosis in the NSLC patients overall. However, in patients with adenocarcinoma, absent Rb protein expression was correlated with the pN factor and showed an association with significantly shorter period of survival after surgery. The analysis of Rb protein expression in adenocarcinomas without altered p53 revealed that absent Rb protein expression was associated with a shorter survival period in these tumours. In our study, decrease of Rb expression showed a significant relation to invasion in multivariate analysis also (Table-56). This indicates that loss of wild-type Rb may play a role in the invasion of this disease. Loss of Rb staining is reported during the progression of Barrett's metaplasia to
dysplasia and adenocarcinoma (Coppola et al., 1999) suggesting the accumulation of unstainable aberrant protein.

**p53**

The p53 tumour suppressor gene is involved in the development and progression of various tumour types (Hollstein et al., 1991). Mutations in this gene, encoding a nuclear phosphoprotein of 393 amino acids, appears to be one of the most common genetic aberrations in human cancers (Greenblatt et al., 1994).

In the present study, an almost similar pattern of expression was noticed for the p53 protein in both normal and molar placentae (Table-25, Plates 10A & B). Usually, but not always, the product of wild-type p53 is undetectable by an antibody in formalin-fixed material because of its short half-life. Thus p53 protein detected by immunohistochemistry has been regarded to reflect a mutant type of p53 (Campo et al., 1991). However, the antibody which we have used recognizes an epitope of both wild-type and mutant p53. A higher expression of p53 in hydatidiform moles compared to early normal placentae and placentae with hydropic changes is reported (Persuad et al., 1993; Chen et al., 1994; Cheung et al., 1994b; Haidacher et al., 1995; Cheville et al., 1996a; Schammel and Bocklage, 1996; Fulop et al., 1998; Qiao et al., 1998). Ozbilim et al. (2000) could not detect significant difference in p53 expression among placentae of spontaneous abortions and hydatidiform moles.
There are previously published studies, demonstrating the absence of p53 gene mutations in GTD (Chen et al., 1994; Cheung et al., 1994b; Shi et al., 1996; Halperin et al., 2000) except the reports of Wu et al. (1997). Therefore, the overexpressed p53 protein seen in this study may also be predominantly of the wild type. p53 expression has been reported in normal trophoblasts, secretory endometrial glands and decidual cells of the stroma (Lee, 1995). The expression of p53 in both normal and molar placentae may be due to the presence of actively proliferating trophoblast cells (Milner, 1991). The wild-type p53 gene is a tumour suppressor with a role in maintaining genetic stability (Greenblatt et al., 1994; Ko and Prives, 1996). The p53 protein is a transcription factor that activates promoters of certain growth-regulatory genes through binding to a specific DNA sequence. Wild-type p53 has also been described as a topoisomerase I binding protein capable of enhancing the cleavage reaction step of the topoisomerase I catalytic cycle in vitro (Gobert et al., 1996). Topoisomerases are necessary for nuclear metabolism, participating in a variety of processes involving the melting and reannealing of DNA including replication, transcription and DNA repair (Anderson et al., 1996). This may account for the expression of p53 in the proliferating trophoblastic population. Muller-Hocker et al. (1997) has also reported an increased expression of p53 in PSTT, a rare form of GTD.

Accumulation of p53 has been correlated with a poor prognosis in nodular and gastric lymphomas (Piris et al., 1994; Nakamura et al., 1996), carcinomas of the oesophagus (Shimaya et al., 1993) and ovaries (Bosari et al., 1993), breast
cancer (Silvestrini et al., 1994) and lung carcinoma (Quinlan et al., 1992). Other studies, however, failed to show such a correlation for lung carcinoma (Brambilla et al., 1993) or pancreatic carcinoma (Di Giuseppe et al., 1994). It has therefore been questioned that p53 will be an independent marker of prognostic significance (Battifora, 1994). The results of the present study also demonstrate a lack of prognostic significance for p53 in complete moles (Table -26).

We have noted occasional cytoplasmic p53 protein. In the literature, mainly nuclear localization of p53 is reported. There are reports suggesting that cytoplasmic p53 represents an inactivated protein, which is logical in view of the fact that one of the main functions of the p53 protein is to act as a transcription factor (Funk et al., 1992). In these cells p53 accumulation in the cytoplasm may have been caused by a failure in the translocation of the protein to the nucleus (Moll et al., 1996). However, we observed no significant association of cytoplasmic staining with the prognosis of the disease and was therefore, not considered later.

Interestingly, a downregulation of p53 protein was observed in the invasive lesions in univariate analysis (Table-27 & Plate 10D), but not in multivariate analysis. In the non-invasive moles, the overexpressed wild-type p53 may be causing a growth arrest while there may be a reduction in expression of the wild-type protein in the invasive moles. However, most p53 alterations involve missense mutations of one allele with loss or rearrangement of the other allele. This suggests that the presence, i.e., a gain of function, of mutated p53 protein, rather
than the loss of wild-type p53 protein, confers a selective growth advantage and promotes clonal expansion (Greenblatt et al., 1994). It has been reported that immortalization is frequently observed in primary cell cultures upon loss of wild-type p53 (Roemer, 1999).

**p21**

Neoplastic diseases are characterized by an unco-ordinated cell growth and the eukaryotic cell cycle is controlled by protein complexes composed of cyclins and cyclin-dependent kinases (CDKs). The activity of CDKs is regulated by association-dissociation with inhibitory subunit designated cyclin-dependent kinase inhibitors (CDKIs) (Maclachlan et al., 1995).

We observed an increased expression of p21 in the molar pregnancies compared to gestational age matched normal placentae, though it was not statistically significant (Table-28). This was in accordance with the previous reports of Cheung et al. (1998) and Fulop et al. (1998) who have reported an increased expression in complete moles and choriocarcinomas than in normal placentae and partial moles. Cheung et al. (1998) detected p21 expression predominantly in the nuclei of the syncytiotrophoblasts. However, in contrast to their findings we could observe p21 expression also in the inner most proliferating basal cytotrophoblast layer of both normal and molar placentae (Plates 11A & C). In the studies of Cheung et al. (1998) p21 was gestational age matched in normal
placentae but not in molar placentae. The elevated amounts of p21 present in the nuclei of molar pregnancies apparently may not have down-regulatory effect on cell growth as predicted for such tumour suppressors. The effectiveness of p21 as a growth inhibitor may be dependent on the levels of cyclin-dependent kinases, which are inhibited by p21 (Neshat et al., 1994). Alternatively, the cell may be attempting to downregulate proliferation through a p21 pathway, but this regulation may not be effective because of the greater effectiveness of competing growth-stimulatory pathways.

A prognostic significance of p21 expression has been reported in some cancers (Gomyo et al., 1997; Ito et al., 1997; Jiang et al., 1997; Ogawa et al., 1997; Wagasuki et al., 1997), whereas other authors have failed to establish any such prognostic value (Ito et al., 1996; Backe et al., 1997; Diab et al., 1997). We did not observe any difference in the different prognostic groups of CHM (Table-29). This is in accordance with the results of Cheung et al. (1998) who have reported a lack of prognostic significance of p21 in molar pregnancies.

A trend of reduced p21 expression in invasive tumours further suggests that p21 may have a role in tumour invasion (Table-30). p21 may mediate growth arrest by inhibiting further DNA synthesis, thus allowing the cells to continue their differentiation. This is substantiated by our present observation of an inverse correlation between p21 and the proliferative markers PCNA and Ki-67 noted in normal placentae (Table-64).
Cell proliferation markers

The proliferative nature of the molar and normal placentae were compared using antibodies to PCNA and Ki-67.

PCNA

Expression of PCNA was seen in the cytotrophoblasts of both normal and molar placentae (Plates 12A & B). This pattern is in agreement with previous studies which have identified the cytotrophoblast as the main site of placental DNA synthesis based on autoradiography with $^3$H-thymidine (Geir et al., 1975). The differential PCNA immunoreactivity in cytotrophoblasts and syncytiotrophoblasts has also been previously reported (Wolf et al., 1992; Molykutty et al., 1998). Mochizuki et al. (1998) have detected PCNA expression in the cytotrophoblasts of normal placentae being most abundant in early placenta, less abundant in midterm placenta and least abundant in term placenta. We also detected maximum PCNA positivity in the first trimester normal placentae compared to second and third trimester normal placentae. However, Persuad et al. (1993) could detect only minimal PCNA positivity in the normal placentae.

Available data on the prognostic value of PCNA and grade of proliferation is documented in various cancers and remain controversial (Haerslev et al., 1996; Sun et al., 1996). In this study no correlation was observed between PCNA and
grade of proliferation. This suggests that PCNA expression is related to the pattern of trophoblastic hyperplasia rather than the degree of trophoblastic hyperplasia which is usually abnormal (Elston and Bagshawe, 1972). However, among the prognostic groups, highest positivity was detected in the chemotherapy group (Table-32 & Plate 12F). Persuad et al. (1993) have detected moderate to intense positivity for PCNA in complete moles, partial moles and choriocarcinomas. However, in the studies of Schammel and Bocklage (1996) PCNA expression could not discriminate between moles and non moles. Whereas, Ozbilim et al. (2000) could distinguish complete hydatidiform moles, spontaneous abortions etc., using PCNA index but not using Ki-67. Mochizuki et al. (1998) could detect only minimal PCNA positivity in the choriocarcinomas.

Mei et al. (1998) reported decreased expression of PCNA in human giant cell lung carcinoma strains with low metastatic potential. PCNA overexpression has been shown to be associated with the extent of invasion of bladder cancer (Boquan et al., 1998). Here, we could detect a double fold increase in the PCNA staining index in the invasive lesions suggesting a link between proliferation and invasion in these tissues (Table -33). However, it lacked significance in multivariate analysis.

**Ki-67**

Ki-67 positivity was detected mainly in the cytotrophoblasts of normal placentae as in the case of PCNA (Plate 13A). The positivity was reduced in the
third trimester indicating decreased proliferation in this stage. A significant inverse correlation between Ki-67 labelling and gestational age in normal placentae is reported (Chan et al., 1999).

Several studies have found the Mib-1 index or Ki-67 labelling index to have potential prognostic value in various malignant disease. A positive correlation between high Ki-67 index and poor prognosis has been reported for upper urinary tract carcinomas (Chowdhury et al., 1996), astrocytomas (Wakimoto et al., 1996) and hepatocellular carcinomas (Ng et al., 1995). However, inconclusive or contradictory results have been reported for certain other tumour types, namely oesophageal squamous cell carcinoma (Youssef et al., 1995; Sarbia et al., 1996), gastric carcinoma (Yonemura et al., 1991; Muller et al., 1996) and lung carcinoma (Pence et al., 1993; Pujol et al., 1996), and several studies showed no predictive value of the Ki-67 index in cervical carcinoma (Cole et al., 1992; Levine et al., 1995; Oka and Arai, 1996).

In our study, the expression of Ki-67 was seen to be higher in the complete moles but it was not statistically significant in the different gestational age groups (Table-34). Jeffers et al. (1996) have also reported strong Ki-67 expression in the villous cytotrophoblast and extravillous trophoblast of the complete moles. In their study, there was no difference in the spontaneously regressing and persisting disease group. The Ki-67 labelling index was however, significantly higher in the extravillous trophoblast in both the groups. We also detected a higher Ki-67
positivity in the cells of the extravillous trophoblasts. According to Schammel and Bocklage (1996) PCNA expression did not discriminate between moles and non-moles while the percentage of rimming cytotrophoblast nuclei reactive for Ki-67 differed significantly between moles and non-moles. But in our study, PCNA was seen to show a better discrimination between the moles and non-moles compared to Ki-67. Cheung et al. (1994a) have reported a lack of significance for Ki-67 in predicting the prognosis of molar pregnancies. According to them, hydatidiform moles that give rise to persistent trophoblastic disease do not have higher proliferative rate than those which do not. According to Cheville et al. (1996b) Ki-67 determined growth fractions may be useful in separating complete moles from partial moles but not partial moles from placenta with hydropic changes. Ostrzega et al. (1998) has shown a more significant difference in the staining pattern between placentae with hydropic changes, partial and complete moles compared to PCNA staining. Shih and Kurman (1998) have reported the use of Ki-67 antibodies along with Mel-CAM antibodies in distinguishing exaggerated placental site, placental site trophoblastic tumour and choriocarcinoma cells. In the present study, Ki-67 labelling index failed to predict the different prognostic groups of CHM (Table-35). However, the positivity showed a significant increase in the invasive lesions (Plate 13D). This significance was seen in multivariate analysis also suggesting that Ki-67 expression can be an independent prognostic indicator of invasion in molar pregnancies (Table-56).
APOPTOSIS RELATED GENES

Apoptosis has been found to play a crucial role in the pathogenesis and prognosis of many human diseases. In the present study, we also evaluated the expression of the apoptosis related genes bcl-2 and caspase-3.

There is some controversy in previously reported studies, dealing with placental apoptosis (Nelson, 1996; Smith, et al., 1997a). Several reports document increased apoptosis in the syncytiotrophoblasts of normal placentae while some others have reported decreased apoptotic rate in the syncytiotrophoblast. Yasuda et al. (1995) and Qiao et al. (1998) have reported a preponderance of apoptosis in the cytotrophoblasts. The studies of Halperin et al. (2000) have reported an increased apoptotic rate in the complete moles compared to the normal placentae by flow cytometric analysis.

The percentage of apoptotic cells in placental tissue stained by TUNEL method also shows wide variation in different studies. Smith et al. (1997a) presented less than 0.2 % of apoptotic cells in placental tissue, while Kokawa et al. (1998a & b) demonstrated up to 30 per cent of apoptotic cells in the same placental tissue. Qiao et al. (1998) has reported less than 1 per cent of apoptotic cells in placental tissue and 2-4 per cent of apoptotic cells in complete mole cases, and also about 30 percent of apoptotic cells in a case of complete mole. Yasuda et al. (1995) have reported the labelling of necrotic cells in addition to apoptotic
cells by the TUNEL method, and this could, in part, account for a possible discrepancy in the incidence of apoptosis, as observed with different techniques.

**bcl-2**

Tumour progression is a multi-step process involving both loss of proliferation control and disruption of programmed cell death (apoptosis). It has been shown that the bcl-2 protein prolongs cell survival in a variety of cells by blocking apoptosis (Hockenbery *et al.*, 1990).

have reported bcl-2 immunoreactivity in the villi syncytiotrophoblasts of individuals undergoing surgical termination of pregnancy and those undergoing miscarriages. Smith et al. (1997a) have reported that placental apoptosis increases significantly as pregnancy progresses, suggesting that it may play a role in the normal development and aging of the placenta. According to Uckan et al. (1997) a differentiation-dependent pattern of bcl-2 expression is noted in the placenta, with the protein being abundant in terminally differentiated trophoblast cells. They have reported an inverse relation between bcl-2 and p53 expression in trophoblast. They have suggested a role for cAMP in the regulation of bcl-2 expression and the expression of bcl-2 in terminally differentiated trophoblast cells may be one mechanism by which the trophoblast mass is preserved during pregnancy. In our study, the expression pattern of bcl-2 did not show much difference in the normal and molar placentae (Table-37). The expression was seen in both the cytotrophoblasts and syncytiotrophoblasts of normal placentae (Plates 14A to F).

Conversely, Uckan et al. (1997) reported relatively low expression of bcl-2 in choriocarcinoma cells rendering them susceptible to apoptosis. Mochizuki et al. (1998) have also observed low bcl-2 and apoptotic signal expression in molar trophoblasts and choriocarcinoma cells. According to Wong et al. (1999) bcl-2 expression is probably regulating apoptosis in normal placenta and GTD whereas bax expression is not. According to them extensive apoptosis was detected in hydatidiform mole compared to spontaneous abortions and normal placentae whereas, the apoptotic index of cases that spontaneously regressed was greater
than those requiring chemotherapy. We have noted an upregulation of the bcl-2 protein in the chemotherapy group, though statistically not significant (Table-38). Deletion mutants and post-translational modifications, such as phosphorylation, are reported to abrogate the normal anti-apoptotic function of bcl-2, whereas the protein may be still detectable by immune reactions (Haldar et al., 1995). Studies of bcl-2 expression in head and neck carcinomas have shown that both positive and negative tumours have been associated with good prognosis (Wilson et al., 1996; Friedman et al., 1997). Conflicting results have also been demonstrated for several other tumour types. In breast and oesophageal carcinomas, bcl-2 expression correlated well with good prognosis (Lipponen et al., 1995; Ohbu et al., 1997). The opposite correlation was found in prostate and testicular carcinomas (Eid et al., 1998; Keshgegian et al., 1998), whereas no such correlation was demonstrated in colorectal carcinomas (Tollenaar et al., 1998). The contradictory results concerning prognosis and expression of bcl-2 protein indicate that induction of apoptosis by proteins of the bcl-2 gene family is very complex and that the influence of individual proteins may vary from tumour to tumour. Accumulated evidence suggest that the proteins of the bcl-2 gene family may interact with each other as homodimers and heterodimers, and that their relative proportions regulate the process of apoptosis (Oltvai et al., 1993; Oltvai and Korsmeyer, 1994; Reed et al., 1995).

We have observed a significant down regulation of the bcl-2 protein in the invasive tumours (Table-39) in univariate analysis. This finding is against
mainstream thinking because the anti-apoptotic action of bcl-2 is expected to confer a survival advantage to the tumour cell. However, bcl-2 has been reported to suppress the proliferative activity of cells, which could explain the less aggressive biology of the bcl-2 positive tumours (Borner, 1996). The decrease in expression of bcl-2 in the invasive lesions was however, not significant in multivariate analysis.

**Caspase**

Apoptotic death is thought to be irreversibly induced once the execution caspases (caspases 3, 6, 7) are activated. Their activation leads to the destruction of a variety of proteins and, moreover, to the activation of destructing enzymes, eg., nuclease. Therefore, the activation of the execution caspases is termed the hallmark of apoptosis. However, according to Marks et al. (1998) the short term inactivation of caspase-3 in vitro, followed by its activation did not result in apoptotic death. In their experiments, irreversibility of its apoptotic action depended on caspase activation for a critical minimum length, varying between minutes and hours, depending on the size of the cell. In the present study, caspase-3 expression was significantly reduced in the molar placentae compared to the normal placentae (Table-40 & Fig. 41). This may be due to a possible impairment of apoptosis in the molar placentae. The studies of Huppertz et al. (1998) have localised pro-caspase 3 in the villous cytотrophoblasts of first and third trimester villi, while the activated caspase showed patchy staining in
restricted areas of the syncytiotrophoblasts. We did not notice a significant association of caspase-3 expression with the prognosis or invasion of the disease (Tables-41 & 42). Therefore, caspase-3 expression may not play an active role in the progression of the disease.

**METASTASIS RELATED GENES**

The expression of the different metastasis related proteins in hydatidiform moles has not been studied in detail. Therefore, we also tried to evaluate the expression of E-cadherin, P-cadherin, CD44, CD44v6 and nm23 in these lesions in comparison to normal placentae.

**Cadherins**

During early pregnancy, cytotrophoblast cells from fetal chorionic villi contacting the uterine wall aggregate into multi-layered columns of non-polarized cells which rapidly invade the maternal tissue (Fisher and Damsky, 1993). In many respects the cell-cell interactions that give rise to the unusual juxtaposition of genetically dissimilar fetal and maternal cells are similar to tumour cell invasion. The nature of human haemochorial placentation so mimics that seen with highly invasive tumours that normal trophoblast has been called ‘pseudomalignant’ (Strickland and Richards, 1992). A great deal of understanding of trophoblast invasion has resulted from studies on tumour cell invasion, and the ability to
penetrate basement membranes is an essential requirement for both tumour and trophoblast cells (Graham and Lala, 1992).

Loss of function of E-cadherin seems to facilitate malignant invasive growth of cancer cells. A decreased expression of E-cadherin has been associated with lack of cohesiveness, higher malignant potential and invasiveness in epithelial tumours of the gastrointestinal tract (Kinsella et al., 1993), ovary (Hashimoto et al., 1989), breast (Gamallo et al., 1993), lung (Williams et al., 1993) and other sites. Germ-line mutations in the E-cadherin gene are found in hereditary gastric cancer (Guliford et al., 1998). E-cadherin expression has been reported in isolated human trophoblasts (Aboagye-Mathiesen et al., 1996). In our study, we observed no difference in the pattern of E-cadherin or P-cadherin expression in normal and molar placentae (Tables-43 & 46).

We noted a down-regulation of the E-cadherin protein in the invasive lesions though statistically not significant (Table-45). E-cadherin has been regarded as a suppressor of invasion in vitro (Vleminckx et al., 1991a & b). It has been consistently demonstrated that down-regulation of E-cadherin occurs concomitantly with acquisition of invasive capacity. The down-regulation is a result of repression of transcription of the gene (Vlemnickx et al., 1991a & b; Brabant et al., 1993). A mutation of the E-cadherin gene would result in a non-functional product. Biochemical modification such as the state of phosphorylation of the intermediate components, catenins and plakoglobin, which link E-cadherin to the cytoskeleton,
or a loss of one of these elements also may be expected effectively to render the cadherin non-functional. P-cadherin is reported to be an indicator of poor survival in breast carcinoma (Solé, 1999). However, our results show that E-cadherin and P-cadherin expression do not play a significant role in the progression of molar pregnancies (Tables-44 & 47).

**CD44**

In many human cancers, markedly increased overall levels have been recognized for CD44, one of the main cell surface receptors for extracellular matrix components (Gunthert et al., 1995). Significant loss of intercellular adhesion molecules, such as cadherins and catenins were reported in conjunction with increased tumour growth and metastatic potential (Smith and Pignatelli, 1997). The expression of CD44 variant isoforms has been shown to be associated with poor prognosis in a wide variety of human malignancies, e.g. colorectal cancer, gastrointestinal lymphoma, non-Hodgkin's lymphoma and cervical cancer (Jalkanen et al., 1991; Joensuu et al., 1993a; Wielenga et al., 1993; Kainz et al., 1995). However, CD44 has been shown to be down-regulated after malignant transformation of certain cell types (Salmi et al., 1993) and the prognostic value of CD44 isoform expression in ovarian and breast cancers is discussed controversially (Joensuu et al., 1993b; Kaufmann et al., 1995; Sliutz et al., 1995; Uhl-Steidl et al., 1995). Goshen et al. (1996) have localised CD44 in the human trophoblasts. In the present study, we observed a statistically significant decrease in the expression of the standard form of CD44 in the complete moles compared
to the normal placentae (Table-49). This shows that a reduction of CD44 may play a role in the development of molar pregnancies. However, it was not related to either the aggressiveness or the invasiveness of the disease (Tables-50 & 51).

Goshen et al. (1996) have reported that in normal placentae, the extravillous trophoblasts express the alternatively spliced form CD44v7-8. According to them these cells retain their invasive capabilities through the permissive extracellular cell membrane by carrying the CD44v7-8 isoform, which binds weakly to hyaluron and prevents it from being degraded by intracellular hyaluronidase. We did not notice the expression of variant CD44v6 in the molar pregnancies (Table-52, Plate 17F). Probably it may not play a significant role in the progression of molar pregnancies.

### nm23

In normal pregnancies the uterus acts to limit implantation. Unlike tumour cells, trophoblast invasion is precisely controlled with spatial and temporal limits of migration under normal conditions (Yang et al., 1993).

The discovery of the nm23 gene and its subsequent identification as a putative suppressor of metastases (Bevilacqua et al., 1989) has elicited a great deal of interest in the mechanisms by which the nm23-H1 protein may suppress the formation of metastatic lesions. The motility-suppressive function of nm23-H1 is associated with histidine-dependent phosphotransferase activity of the molecule (Freije et al., 1997). nm23 expression has been shown to be elevated in several
different tumours of lower metastatic potential, including melanoma, breast, hepatocellular, ovarian, lung and gastric carcinomas, whereas in other tumours, such as neuroblastoma and pancreatic carcinoma, the opposite trend has been reported (Rosa et al., 1995; Mei et al., 1998). In our study, nm23 expression showed an increased but not statistically significant expression in molar placentae compared to normal placentae (Table-53 & Plate 18F).

Our results showed no significant association with the prognosis of the disease (Table-54). However, there was a reduction in the expression of nm23 in the chemo group compared to the spontaneously regressing group. From the results of the study it is obvious that nm23 expression showed a significant reduction in the invasive lesions compared to the non invasive ones. (Table-55). nm23 H1 could be one of the factors involved in suppressing early stages of metastasis, such as invasion and migration, as it has been shown to suppress motility and colonization in melanoma and breast carcinoma cell lines (Leone et al., 1991; Freije et al., 1997). Overexpression of nm23 is reported to participate in carcinogenesis and the reduction in nm23 is involved in metastasis and invasion in gastric, colorectal and bladder cancers (Tahara et al., 1993; Boquan et al., 1998).

VIRUSES

Oncogenic activity has been described for several human viruses and worldwide, viral infection is thought to contribute to up to 20% of all human cancers (Vousden and Farrel, 1994).
Based on published reports and the hypothesis that viral infection could be an etiological factor in GTD, association of viruses was evaluated in CHM and normal placentae. The study on HIV was based on a report by Zachar et al. (1991), suggesting permissiveness of transformed trophoblast cells to HIV. In Western countries, HIV seroprevalence among child bearing women is generally under 1% whereas in developing countries, the seroprevalance among pregnant women can be as high as 32% (De-Ruiter and Brocklehurst, 1998). Even though situated in a developing country, Kerala has a low HIV prevalence (Legori et al., 1998). In Thiruvananthapuram, the prevalence rate of HIV is 10/100,000 individuals and in pregnant women this is about 5/100,000 pregnancies (Personal Communication-AIDS Cell, Medical College, Thiruvananthapuram, Kerala). Our results support the low prevalence rate of AIDS and also suggest that HIV infection cannot be considered an etiological factor in GTD (Enose et al., 1998).

The results of our study on CMV and HSV suggest no increased incidence or chronicity of these viral infections in CHM and their roles as etiological factors of this disease can be ruled out.

Human papillomavirus (HPV) infection is reported in benign and malignant tumours in a variety of body sites, such as the uterine cervix and anogenital tracts (Arends et al., 1990). In head and neck regions, HPV types 6b or 11 DNAs are detected in more than 90% of juvenile and adult-onset papillomas, while the viral genomes including oncogenic types are also demonstrated in 15-38% of malignant
lesions (Arends et al., 1990; Caruana et al., 1997; Paz et al., 1997). The results of the present study obviously suggest an etiological role for HPV infection with the pathogenesis of CHM (Plate 20B). HPV infection is usually prevalent in spontaneous abortion specimens and Pao et al. (1995) have reported the presence of HPV 18 DNA in hydatidiform moles and choriocarcinomas.

**VDRL Test**

The prevalence rate of syphilis in the population in Thiruvananthapuram is 200/100,000 individuals and among pregnant women, the prevalence rate is 30/100,000 (Personal Communication-AIDS Cell, Medical College, Thriuvananthapuram, Kerala). In our study, only one sample of all the samples tested was positive for syphilis antibody. Our results support the low prevalence rate and also suggest no association of syphilis infection with the etiology of GTD (Enose et al., 1998).

**MULTIVARIATE ANALYSIS**

On carrying out a multivariate analysis of the parameters that showed significance in univariate analysis, TGF-α expression and gestational age emerged as independent prognostic indicators of persistence of the disease (Table-56). This suggests that TGF-α may play an active role in trophoblastic proliferation. The fact that better prognosis was observed in the group of patients that presented with the disease in the late gestational ages is reasonable, since the late presentation itself may be due to decreased aggressiveness.
As indicated earlier, among the parameters that were analysed by multivariate analysis to identify invasive cases at an early stage, decrease in Rb expression and increased Ki-67 expression were significant variables (Table-56). In the invasive lesions, a decreased expression of the tumour suppressor genes studied were noticed and among them loss of Rb emerged out to be statistically significant (Plate 9F). Increased Ki-67 expression indicated increased proliferation in the invasive tumours (Plate 13D). Thus these parameters could be of help in identifying the aggressive lesions at an early stage and would be of help to the clinicians in deciding the chemotherapeutic treatment regimen.

**CROSS CORRELATIONS**

The variables that showed significance at the level of P < 0.005 in bivariate analysis were considered to look into their difference in synergistic/antagonistic actions in normal placentae and molar placentae.

**Correlative analysis of Growth factors with Growth factor receptors**

Cell growth or its inhibition is largely controlled by autocrine and paracrine interactions of growth factors with their receptors. The malignant cell transformation and growth requires concurrent expression of compatible growth factor-receptor combinations, growth stimulation by addition of ligand or growth inhibition by neutralizing antibodies. We analysed the correlation between the
growth factors and receptors to look into their mechanisms of action in both the tissues (Table -57).

**EGF vs TGF-α** - A significant positive correlation between the growth factors EGF and TGF-α was noticed in both normal placentae and molar placentae. The possible reason is that both these growth factors are expressed in a gestational age dependent manner in both the tissues. Joiakim *et al.* (1994) have demonstrated that induction of transformation by EGF in Ad12-SV40 immortalized epithelial cells was accompanied by stimulation of TGF-α (200%) and EGFR mRNAs (100%). TGF-α antisense oligomers were found to inhibit the growth of immortalized cells indicating that EGF induction of transformation is mediated by increased levels of TGF-α mRNA. Thus, the expression of EGF may in turn also cause the stimulation of TGF-α in the trophoblast tissue also. Pedersen *et al.* (1994) demonstrated that EGF is a more potent stimulator compared to TGF-α in human glioma cell line. As discussed earlier, in our study by univariate analysis both EGF and TGF-α showed relation with prognosis of the disease and EGF with invasion, while in multivariate analysis TGF-α emerged as the prognostic marker of persistence of the disease (Table-56).

**EGFR vs c-erbB-2** - The overexpression of the receptors EGFR and c-erbB-2 seem to correlate in both the tissues. The first identified member of the type I receptor tyrosine family of receptors, the EGFR, is a transmembrane glycoprotein that mediates the mitogenic response to the EGF family of ligands that includes
both EGF and TGF-α (Carpenter, 1987). On activation, the EGF receptor phosphorylates tyrosine residues on its C-terminal tail and has been shown to interact with other members of the RTD-1 family like c-erbB-2, cerbB-3 and c-erbB-4. These intracellular phosphorylations can initiate signalling cascades which eventually result in gene activation (Egan and Weinberg, 1993). The activated EGFR may interact with and transphosphorylate the c-erbB-2, and the heterodimerisation mechanism may be active in both normal placentae and molar placentae and may contribute to their proliferation. Nicholson et al. (1993) have reported that a functional interaction between these proteins may contribute to aberrant growth of a subset of breast tumours.

**EGF vs EGFR.** EGF and EGFR expression were related both in normal and molar placentae. Growth factors bound to EGFR are able to stimulate the growth of tumour cells in an autocrine or paracrine manner. According to Chen et al. (1988) EGF-binding sites and EGFR production increase in human placentae throughout the gestational period. In the present study, there is expression of EGFR in both normal and molar placentae and the overexpression of EGFR with EGF seem to relate more significantly in molar placentae. This again points towards a more active autocrine mechanism for EGF induced proliferation in molar placentae. The binding of EGF to the specific cell membrane receptor EGFR results in phosphorylation of receptor-associated protein and an increased membrane phospholipid turnover, followed by clustering and internalization of the epidermal growth factor receptor complex. During this process, biochemical signals are
initiated, leading to RNA and protein synthesis which, in turn, promote cell division. The relation between EGF and EGFR suggest that these mechanisms are active in these tissues.

**TGF-α vs EGFR** - TGF-α shows a positive correlation with EGFR both in normal and molar placentae. This again shows an autocrine mechanism active in both the tissues. EGFR and its ligands appear to play a very critical role in the pathogenesis and progression of tumours. TGF-α is reported to be co-expressed with EGFR in a variety of carcinomas (Murray *et al*., 1993; Wang *et al*., 1997). *In vivo*, between 20 to 60% of EGFR-expressing tumours would also express TGF-α, suggesting that an autocrine loop may be involved in the growth of such tumours. The markers were almost invariably located within the cytoplasm, which might suggest their crucial role in growth regulation and cell differentiation. In gliomas, TGF-α and EGFR function as an important autocrine loop in supporting cell proliferation (Tang *et al*., 1997). The EGFR autophosphorylation could create binding sites for SH-2 containing signalling molecules and activate multiple signalling pathways (Songyang *et al*., 1993). Thus the relation between TGF-α and EGFR indicates the mitogenic action of this growth factor through EGFR in the trophoblastic tissue. The significance of TGF-α with EGFR was stronger than EGF-EGFR relation in CHM indicating that TGF-α may play a more active role in the trophoblastic tissue.

**EGF vs c-erbB-2, TGF-α vs c-erbB-2** - These relations were observed in molar placentae and not in normal placentae. It has been suggested that the function of
c-erbB-2 may not be to bind to any ligand directly, but rather to increase the binding affinity of ligands to heterodimers of the 

erb family (Karunagaran et al., 1996). c-erbB-2 can form heterodimers with the EGFR and neu differentiation factor receptors erbB-3 and erbB-4. The overexpression of c-erbB-2 in cell lines have shown to reduce the dissociation rate of EGF and NDF resulting in increased activation of measured cytoplasmic kinases. c-erbB-2 can affect the response to a wide variety of ligands by altering the binding affinities of growth factor receptors, and its overexpression may result in a growth advantage for affected cells (Houston et al., 1999). This may account for the significant correlation observed between the growth factors EGF and TGF-α with c-erbB-2 in molar placentae. Moreover, as indicated above EGFR shows relation with both EGF and TGF-α in molar pregnancies that show an increased expression of c-erbB-2 indicating that the ligand binding affinity of EGFR may be increased in the molar placentae expressing an increased amount of c-erbB-2 thereby contributing to its proliferation.

**Correlative analysis of Growth factors and Receptors with Signal transducers and Tumour suppressor proteins**

Since the mitogenic action of the growth factors through the receptors requires the transmission of the signals to the cytoplasm and nucleus, thereby affecting proliferation the association between these proteins were studied. Since the alteration in the cell cycle mechanisms caused by these growth factors and receptors may also regulate the expression of the regulatory molecules such as
tumour suppressor proteins, their association was also looked into (Table-58).

**EGF vs ras, c-erbB-2 vs c-myc, c-erbB-2 vs ras** - In the regulation of cell proliferation, positive growth factors like EGF and TGF-α on binding to cognate receptors result in dimerization and autophosphorylation (or cross phosphorylation on tyrosine residues). The phosphorylated tyrosine can serve as docking site for cytoplasmic enzymes or adaptor molecules. The enzymes become phosphorylated and activated and the adaptor molecule activates ras signalling to activate raf which in turn activate MAP kinase pathway culminating in modulation of transcription factors necessary for initiating progression through the early G1 phase of the cell cycle. The c-myc and p21ras oncogenes usually promote cell proliferation in the presence of growth factors, but in their absence the cells undergo apoptosis (Evan *et al.*, 1992; Tanaka *et al.*, 1994). In the present study, in normal placentae EGF was related to ras oncprotein and c-erbB-2 was related to c-myc oncoproteins. This again suggests that the EGF activation of EGFR and the transphosphorylation of c-erbB-2 may in turn activate the ras and c-myc signal transducers thereby affecting cell proliferation in these cells. c-erbB-2 overexpression appeared to stimulate ras oncogene expression both in the normal placentae and CHM lesions suggesting that consequences of up-regulated receptor kinases lead to ras activation and cell growth. In human breast cancer cell lines, c-erbB-2 overexpressed cells showed a constitutive phosphorylation of Shc that led to ras activation (Stevenson and Frackelton, 1996). Flow cytometry studies by Schackney *et al.* (1995) have shown that in aggressive breast cancer cells c-erbB-
2 overexpression is associated with ras overexpression. A possible interaction between c-erbB-2 and ras in cellular transformation is suggested by two independent considerations: first, c-erbB-2 belongs to the tyrosine kinase growth factor receptor family, some members of which interact with rasp21 via GAP (Ellis et al., 1990; Kaplan et al., 1990), second a factor enhancing the autophosphorylating activity of p185 was found in the conditioned media from ras-transformed cells (Yarden and Weinberg, 1989). Enhanced ras expression is reported to co-operate with c-erbB-2 activation to endow breast cancer cells with a particularly aggressive phenotype (Dati et al., 1991).

TGF-α vs p53, EGFR vs p53, c-erbB-2 vs p53, c-erbB-2 vs Rb - We observed a correlation between TGF-α and p53 in the normal proliferating tissues. However, this mechanism was absent in the molar tissues. Overexpression of TGF-α is reported to exhibit accelerated growth rate and increased expression of cell cycle related genes like p53 resulting in increased cell cycling (Tan et al., 1994). A positive correlation between the expression of p53 and EGFR was also evident in normal placentae. The reports of Deb et al. (1994) show that wild-type human p53 transactivates the human EGFR promoter in Saoss-2 osteosarcoma cells in a dose-dependent manner. This suggests that increased wild-type human p53 concentration may induce the expression of EGFR. Overexpression of wild-type human p53 results in arrest of cells in the late G1 phase of the cell cycle and may also lead to apoptosis. The characteristics of p53 reported so far deal predominately with its growth inhibitory function. Interestingly, growth promoting
role for wild type p53 has been reported (Shekhar et al., 1995). Wild-type p53 activates the EGFR and thereby may induce EGFR expression. Thus, it is suggested that wild type p53 may act synergistically with EGF in enhancing the cell proliferation, at least in certain cell types. This synergy may result in proliferation of cells originally expressing few or no EGFR. Increased EGFR concentration is observed in various forms of cancers (Wells, 1999). This suggests in a provocative way that wild-type p53 may be involved in hyperproliferation of cells. This may confer a growth advantage to this rapidly growing normal placental tissue and may also represent a mechanism of cell growth regulation (Ludes-Meyers, 1996). The activation of the EGFR promoter by wild-type human p53 may also suggest the possible existence of a regulatory mechanism to control cell proliferation. Perhaps due to signals like DNA damage (Kastan et al., 1991), if the p53 concentration becomes relatively high to throttle cellular growth, either by growth arrest or apoptosis (Debbas and White, 1993), a mechanism to increase cellular EGFR concentration may work to drive the cell to a balanced path of moderate cell growth. The correlation between p53 and EGFR in normal placentae but not in CHM suggests a mechanism of growth regulation in normal placentae which may be absent in CHM. Similarly, in the present study, c-erbB-2 was related to p53 only in normal placentae and not in molar placentae. Nielson and Nyholm (1994) observed that in endometrial carcinomas c-erbB-2 immunostaining did not correlate with p53 immunostaining. A correlation between c-erbB-2 and Rb proteins is also noticed in the normal tissues. The tumour suppressor gene Rb has been shown to be able to suppress c-erbB-2 induced transformation of cells
by repressing c-erbB-2 transcription (Yu et al., 1992).

**Correlative analysis of Growth factors and Receptors with Proliferation markers**

The proliferative action of the growth factors and receptors were assessed by their association with the proliferative markers (Table-59).

**EGF AND TGF-α vs PCNA, EGF, EGFR AND c-erbB-2 vs Ki-67:**

EGF was related to PCNA in normal placentae, not in CHM and TGF-α to PCNA in molar placentae and not in normal placentae. Autocrine and paracrine growth factors are shown to modify mRNA stability and hence PCNA accumulation (Hall et al., 1990). As suggested earlier TGFα-EGFR system appear to be more active in CHM. We observed a significant positive correlation between EGFR and Ki-67 in both normal and molar placentae and c-erbB-2 to Ki-67 in molar placentae. A relation between the expression of growth factors and receptors with proliferation is reported in different cancers (Nicholson et al., 1994; Oehler et al., 1997). All these demonstrate the mitogenic effects of these growth factors through activation of their receptors. These mitogenic effects may contribute to their significance with prognosis and with invasive nature of the tumours.

**Correlative analysis of Growth factors and Receptors with Metastasis related proteins**

The relation of the growth factors and receptors with metastasis related proteins was studied (Table-60).

**EGF vs CD44, c-erbB-2 vs E-cadherin** - Growth factors co-ordinately regulate a variety of genes associated with pathological states including tumour invasion and
metastasis. Overexpressed EGFR on tumour cell surfaces is associated with enhanced cell attachment and migration into extracellular matrices, which promotes tumour aggressiveness. In the present study, we have observed a correlation between EGF and CD44 in normal placenta but not in CHM. It has been reported that EGF up-regulates the cell surface adhesion molecule CD44 at both the mRNA and protein levels on mouse fibroblasts expressing full-length wild-type EGFR (Zhang et al., 1997). We observed a significant negative correlation between c-erbB-2 and E-cadherin in normal placentae but not in molar placentae. Down-regulation of E-cadherin expression in vitro has been reported to occur by the activation of the oncogene product c-erbB-2 (d'Souza and Taylor-Papadimitriou, 1994). Thus, these mechanisms seem to be impaired in the molar placentae.

**Correlative analysis of Signal transducers with Tumour suppressor proteins and Apoptosis-related proteins**

The relation of the signal transducers with tumour suppressor proteins and apoptosis related proteins was studied (Table-61).

**Ras vs Rb-** The ras proto-oncogene is a central component of mitogenic signal transduction pathways, and is essential for cells both to leave a quiescent state (GO) and to pass through the G1/S transition of the cell cycle. The innermost bound protein ras integrates various extracellular signals that are subsequently communicated from the cytoplasm to the nucleus via the Raf/MEK/MAPK cascade. Retinoblastoma protein Rb, previously reported to be a nuclear target of this pathway, can in turn influence
the activation state of Ras. Rb deficient fibroblasts display elevated levels of activated Ras during G1 phase. Bidirectional nuclear cytoplasm communication between Rb and Ras is reported (Lee et al., 1999). Thus a functional crosstalk between Rb and ras protein which balances the cell phenotype between normal and transformed states is reported (Kivinen et al., 1993). Expression of normal, but not mutant Rb protein in mouse fibroblast cells were shown to prevent c-Ha-ras oncogene mediated cellular transformation and colony formation. Peeper et al. (1997) have reported that Rb is an essential G1-specific mediator that links Ras-dependent mitogenic signalling to the cell cycle regulation. The effect of ras overexpression on the Rb gene was significant only in the molar placentae. This again shows that mechanisms might exist in normal tissues to counteract the effect on cell cycle regulatory genes which might be impaired in CHM.

**c-myc vs bcl-2** We have observed a positive correlation between the expression of c-myc and bcl-2 in the molar placentae. The apoptotic pathway may serve as a mechanism to protect an organism from uncontrolled cell cycle and tumourgenesis induced by inappropriate c-myc expression. Therefore, cells in which c-myc expression is deregulated may not necessarily develop into tumours and instead can be eliminated by apoptosis unless a second event that blocks the apoptotic pathway occurs. These cells can be rescued from apoptosis by co-expression of 'survival genes' such as bcl-2, or by loss of critical components of the apoptotic pathway, such as p53. Wild-type p53 activity is essential for c-myc induced apoptosis in a manner independent of induction of p53 target proteins such as p21. Overexpression of bcl-2 or inactivation of p53 each independently seen in tumours with c-myc deregulation, thus can inhibit the c-myc mediated cell death pathway, and may be the mechanisms by which myc
deregulation can lead to proliferation instead of programmed cell death (Wagner, 1995). The inactivation of p21 and related cdk-inhibitors may explain several of the oncogenic actions of c-myc, including the induction of proliferation, immobilisation and inhibition of differentiation (Hermeking, 1995). Additive effects or complementation of bcl-2 expression with the expression of other oncogenes, such as c-myc have been reported. For instance, Marin et al. (1995) has shown that double transgenic c-myc/bcl-2 mice rapidly developed lymphomas. Thus the correlation of c-myc with bcl-2 may play a role in the proliferation of CHM.

**ras vs bcl-2** - We have also noticed a positive correlation between bcl-2 and p21ras in both normal placentae and CHM. As mitochondria and endoplasmic reticulum are both sites of calcium storage, bcl-2 is thought to influence calcium regulation. Calcium-dependent nuclease is reported to be involved in the DNA fragmentation seen in apoptosis (Caron-Leslie et al., 1991). Thus, bcl-2 may be involved in redistributing or sequestering calcium, thereby decreasing nuclease activity and inhibiting apoptosis. In different cells, bcl-2 either has an effect or has no effect on the overall level of intracellular free calcium (Baffy et al., 1993; Zhong et al., 1993). Calcium can function in intracellular signalling, and bcl-2 could also affect other signal transduction pathways. This is suggested by the finding that bcl-2 interacts with ras (Fernandez-Sarabia and Bischoff, 1993).

**Correlative analysis of Tumour suppressor proteins**

The interactions between the tumour suppressor protéines was also looked into in the present study (Table-62).

**Rb vs p53** - Since both Rb and p53 genes are associated with the progression of cells into the S-phase of the cycle, a co-operative effect of both these genes have been
suggested in cell cycle regulation. The discovery of inhibitors of CDK activity has highlighted the possibility that the p53 tumour suppressor protein may itself be indirectly regulating the function of Rb protein. The p53 induced transcriptional activation of the p21-CDK-inhibitor (El-Deiry et al., 1993) result in the inhibition of cyclin E/CDK2 kinase activity (Dulic et al., 1994) and prevents Rb from being released from E2F-1. The net result is the continuation of Rb-induced repression of E2F-1, leading to inhibition of S-phase progression. p53 appears to be able to regulate the transcription of the Rb gene itself and two regions of the gene have been identified: one of these can stimulate transcription from Rb promoter and the second one represses Rb transcription (Osifchin et al., 1994). We have observed a significant correlation between p53 and Rb in normal placentae but this co-operative effect seems to be lacking in the molar placentae probably playing a role in this condition.

It is reported that p53 regulates p21 expression (El-Deiry et al., 1993; Li et al., 1994; Harper et al., 1993), however, our study found no statistical correlation between p53 and p21. The lack of p53-p21 correlation also has precedence in the literature (Gomyo et al., 1997), and probably relates to p53-independent pathways of expression. The elevated p21 expression most likely reflects upregulation from p53 independent pathways (Michieli et al., 1994). Cheung et al. (1998) have also reported the operation of p53 independent pathways in GTD.

**Tumour suppressor proteins and apoptosis related proteins**

The relation of the tumour suppressor proteins with apoptosis related proteins was studied in both normal placentae and molar placentae (Table-63).

**p21 vs bcl-2** - In our study significant relation between p53 and bcl-2 was absent
in both normal and molar placentae, while p21 showed a significant negative relation with bcl-2 in both the tissues. In normal cells, there is a complex molecular network by which programmed cell death and cell replication are integrated. Wild-type p53 is known to play an important role in causing cell-cycle arrest in damaged cells in the G1 phase and in the induction of apoptosis (Smith and Fornace, 1996). This effect may at least be partially mediated by bcl-2 as p53 is capable of downregulating the bcl-2 gene as well as inducing expression of bax, a bcl-2 related protein with apoptosis-promoting properties (Miyashita et al., 1994). A recent report by Halperin et al. (2000) has suggested a role for p53-dependent apoptosis to modulate excessive trophoblastic proliferation in the pathogenesis of GTD. However, in contrast to other investigators (Barbareschi et al., 1996a; Bukholm et al., 1997; Elledge et al., 1997; Hori et al., 1997; Jensen et al., 1997; Nakopoulou et al., 1996), we failed to detect any relationship positive or inverse, between the two immunomarkers examined. The contribution of bcl-2 protein in pathogenesis of nasopharyngeal carcinoma, breast cancer and bronchioloalveolar carcinoma is also reported to be independent of p53 protein (Murono et al., 1999; Mc Donald and Pilgram, 1999; Nakopoulou et al., 1999). The lack of correlation implies that regulation of bcl-2 expression could be totally independent of p53 or that wild type p53 would have a minor or secondary role in the regulation of the expression of bcl-2 in normal placentae and CHM. The highly complex structure of the normal bcl-2 gene indirectly suggests that several levels of control and a host of regulatory factors may be involved in its expression. In other articles (Upadhyay et al., 1995; Caffo et al., 1996; Bukholm et al., 1997), bcl-2 expression was again not associated with the p53 protein level itself, but with the p53 induced suppression of p21 mRNA expression. This interaction may be a mechanism
by which bcl-2 exercises its oncogenic potential. In our study, a negative correlation was observed between p21WAF1/CIP1 and bcl-2 in both normal placentae and CHM. This supports the reports of Upadhyay et al. (1995) in breast epithelial cells that bcl-2 suppresses p21 expression.

**Correlative analysis of Tumour suppressor proteins with Proliferation markers**

The growth suppressive function of the tumour suppressor proteins was studied by looking into their significant relations with the proliferation markers (Table-64).

**p21 vs PCNA** - By participating in the inhibition of the PCNA dependent and E2F1-dependent G1-S transit, the tumour suppressor proteins are indicated to uncouple growth from differentiation allowing entry into the maturation path. Where this function of the tumour suppressor proteins is impaired, sustained proliferation at a stage of incomplete maturation is expected to ensue, that condition being a prime characteristic of the cancer cell. Deregulation of cell proliferation and differentiation is important in neoplastic transformation. p21WAF1/CIP1 is known to be related to the control of proliferation and differentiation of cells. In normal cells, p21 exists in quaternary complexes with cyclin, CDK and PCNA. p21 can induce G1 arrest and block entry into the S phase by inactivating CDKs or by inhibiting the activity of PCNA. On the other hand, induction of p21 expression has been demonstrated during differentiation of various cell types, both during embryological development (Halevy et al., 1995), and in *in vitro* experiments (Steinman et al., 1994). Although the expression of p21 varies among different human tissues, it occurs mainly in quiescent
cells (Fredersdorf et al., 1996). For example, p21 WAF1/CIP1 immunoreactivity in colonic normal mucosa and adenomas was seen in the superficial third of the crypts (maturation compartment) and in surface (terminally differentiated) epithelium (Doglioni et al., 1996). Thus, p21 WAF1/CIP1 may be important in the maintenance of growth arrest in terminally differentiated cells by inhibiting DNA synthesis. p21 and the proliferative index PCNA showed a negative correlation in normal placentae. According to Xiong et al. (1993a) and Yook and Kim (1998), stoichiometry of p21WAF1/CIP1 and CDK complexes may regulate its activity. When the ratio of p21 to the complex is more than one, p21 inhibits the kinase activities. If the ratio is less than one, p21 serves only as an assembling factor of cdk complex and does not inhibit cdk activity (Waga et al., 1994; Zhang et al., 1994). It is reported that in contrast to the growth arrest in a normal cell, the proliferation index is not related to the p21WAF1/CIP1 expression in ovarian cancer cells (Barboule et al., 1995). Similarly, PCNA was not related to p21 expression in CHM.

**p21 vs Ki-67** - As p21WAF1/CIP1 negatively regulates cell cycle progression, an inverse correlation between p21 expression and proliferative activity would be expected. Such a negative correlation between p21 and Ki-67 was observed in normal placenta. In CHM, the pattern was complex. At the single cell level, p21WAF1/CIP1 and MIB-1 immunoreactivity were observed in the cytotrophoblasts. A mutually exclusive pattern involving p21WAF1/CIP1 and Ki-67 expression has been reported in colonic (Doglioni et al., 1996) and gastric tissues (Yasui et al., 1996). p21WAF1/CIP1 expression in early placentas, which display more active trophoblastic proliferation, is higher than in mature placentas, where the proliferative activity is lower.
(Boyd, 1984; Cheung et al., 1993b; Cheung et al., 1994a). In the present study, statistically significant direct correlation between p21WAF1/CIP1 expression and proliferative activity could not be established in complete hydatidiform moles. This lack of correlation between p21WAF1/CIP1 expression and the proliferative (Ki-67) index has also been reported in cancers of the stomach, colon, breast and ovary (Doglioni et al., 1996; Yasui et al., 1996; Barbareschi et al., 1996b; Barboule et al., 1995; Werness et al., 1997; El-Deiry et al., 1995). Yasui et al. (1996) suggested that tumour cells might have escaped terminal differentiation and growth arrest by becoming refractory to the inhibitory signals from p21WAF1/CIP1 (Yasui et al., 1996). Moreover, proliferation and progression of trophoblastic tissues may be regulated by factors other than p21WAF1/CIP1. p21 negatively regulates cell proliferation by inhibiting Cdns in some normal tissues (El-Deiry et al., 1995) and tumour cells (El-Deiry et al., 1993). The present demonstration of an inverse correlation between p21 and proliferation marker Ki-67 is in alignment with this notion (El-Deiry et al., 1993, 1995).

**Correlative analysis of Tumour suppressor proteins, Signal transducers, Apoptosis related proteins and Proliferation markers with Metastasis related proteins**

The association of the metastasis related proteins with the cell cycle regulatory genes was also studied (Table-65).

**Ras, Rb, p53, bcl-2, PCNA AND Ki-67 vs nm23**: In normal placentae, as well as in complete moles, nm23 expression showed significant relation with the expression of ras, Rb, p53, bcl-2, PCNA and Ki-67 expression. An association between the level
of nm23 has been found in several human tumours, such as neuroblastoma (Hailat et al., 1991), prostrate carcinomas and in leukemia (Igawa et al., 1994). Many potential roles have been suggested for NDPK, including regulation of cellular processes depending upon the nucleotide triphosphate pool, such as DNA synthesis, G-protein mediated signal transduction and tubulin polymerization (Cipollini et al., 1997). In this study the relationship of nm23 with ras in hydatidiform moles also indicates the association of NDPK with G binding proteins and the regulation of function of G proteins in signal transduction. A diverse set of data suggest that phosphorylation of Rb is a critical regulator of its growth-suppressing activities during the progression of the cell cycle. The regulatory activity of Rb in the cell cycle, might be regulated at the post translational level, possibly by phosphorylation and dephosphorylation events carried out by cell cycle-dependent Rb kinases and phosphatases. The NDP kinase activity of nm23 may also possibly have a role here. nm23H-1 is seen to be expressed continuously throughout the cell cycle, higher expression being present in late G1, early S and G2-M phases (Igawa et al., 1994), and has been related to the proliferative phase of cell growth. In the present study, also nm23 was related to the expression of both the proliferation markers PCNA and Ki-67 and to p53 and bcl-2. These correlations suggest a functional contribution of this protein in cell proliferation in the normal tissues.

**Correlative analysis of HPV infection with the expression of Cell cycle regulatory proteins**

The results of our study on HPV suggests that the prevalence rate of HPV infection in CHM is higher than that in normal placentae probably with a higher degree of amplification in CHM. Therefore, the significant relation of HPV infection with the
expression pattern of the different cell cycle regulatory proteins was also studied (Table-66). HPV E6 and E7 oncoproteins are reported to be capable of functionally inactivating the cell cycle regulators, such as p53, Rb and cyclin D1 resulting in the disruption of the normal cell cycle control (Scheffner et al., 1994; Lukas et al., 1994). Yasumoto et al. (1991) have reported the downregulation of HPV-16 E6/E7 mRNA by EGF in human keratinocyte cell line. Woodworth et al. (1992) has shown that both high risk and low risk HPV E7 proteins are capable of inducing expression of proliferating cell nuclear antigen (PCNA) and reactivating cellular DNA replication of the suprabasal, differentiated cells of epithelial raft cultures. However, the positivity to HPV immunostaining or the amplification status showed no correlation with the expression of the genes such as c-myc, PCNA, Ki-67 or suppressor genes such as p53, Rb and p21 in normal placentae or CHM. HPV infection, however, showed a positive correlation with the expression of EGFR, E-cadherin and CD44v6 in normal placenta. No such correlations were noticed in CHM. These observations suggest that the mechanism of action of HPV proteins in normal placenta and CHM can be different and HPV infection probably does not affect the proliferative, apoptotic or tumour suppressor compartment in CHM. These findings need further validation and confirmation to see the real difference in action, if any.