SUMMARY AND CONCLUSIONS

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Gestational trophoblastic disease (GTD) is a spectrum of interrelated diseases arising from the placental trophoblast with a wide range of biological behaviour (Altieri, 2003). This disease occurs predominantly in the reproductive age group with a high prevalence rate in the State of Kerala (1.2% of deliveries). A delay in the diagnosis of persisting disease may increase the patient's risk of developing malignant GTD. The knowledge of precise molecular changes that are critical to pathogenesis remains unknown. Since in GTDs there is increased propensity for abnormal proliferation, survival and accumulation of trophoblastic cells, and EGFR is a molecule involved in these functions; the integrity of the EGFR signal transduction pathway might be a deciding factor. Aberrant EGF receptor signalling due to over-expression, gene-amplification or mutations has been frequently implicated in hyper-proliferative diseases such as cancer. It has been demonstrated that the phosphatidylinositol 3-kinase-Akt/PKB and the Ras-MAPK cascades are major downstream pathways of activated EGFRs mediating resistance to proapoptotic stimuli (Samanta et al, 2004; Franke et al, 2003). We present here the first comprehensive study addressing the profiles of EGF receptor alterations and associated altered signal transactivation pathways in GTDs (107 cases; and 68 gestational age matched normal placenta); and their biologic and prognostic significance.

The expression level of EGFR was evaluated immunohistochemically using antibodies against its intracellular domain (ICD) and extracellular domain (ECD), and confirmed by western blot analysis of the same samples. The phosphorylation status of the EGFR protein was assessed by ELISA. The amplification of the EGFR gene was screened for by differential PCR. The samples that showed over-expression of the EGFR protein were subjected to sequence analysis of the exons 2-7 for mutations in the extracellular domain coding region. Immunohistochemical analysis of the Akt and Ras protein expression levels was done. The apoptotic index was assessed in H&E stained slides and by TUNEL assay and results confirmed by laddering assay. The expression profiles of the apoptotic-cascade proteins Bax, Caspase-3 and Bcl-2 was assessed immunohistochemically. The serum level of EGFR-ECD was assessed by ELISA technique. Statistical analysis using non-parametric Mann-Whitney U Wilcoxon rank sum test, $\chi 2$ tests and bivariate correlation analysis was carried out for evaluation of significant alterations and correlations.

In comparison with normal placenta, EGFR-ICD expression was significantly increased in GTD ($p<0.001$), showing significant correlation with disease persistence. A decreased level of EGFR-ECD observed in the persistent disease group was not

statistically significant. The immunohistochemical observations were duplicated and confirmed in the western blots of the same samples. These findings implicate the EGFR to provide a growth advantage to the trophoblasts, and the likely presence of receptors with molecular alterations.

Assessment of the phosphorylation status of EGFR protein revealed that 55% of the GTD cases ($p<0.001$), of which 62.5% were with persisting disease, exhibited significantly higher levels of phosphorylation. The active role of EGFR in GTD is reiterated.

On screening for the presence of gene amplification, the molar placenta, especially those in the persisting disease group, evidenced significantly increased EGFR gene copy numbers ($p<0.001$); co-presenting with significantly elevated protein expression and phosphorylation.

In this study screening for mutations in exons 2-7 of the extracellular domain of EGFR revealed the presence of in-frame insertion mutations in exon 3, and substitution mutations in exons 4, 5, 6 and 7, only in GTD cases. 75% of the persisting disease group exhibited presence of mutations, of which slow regressing cases represented coupled mutations in exon 3 and 4, whereas cases that had to be treated with chemotherapy showed coupled single mutations in exons 4 and 6. The spontaneously regressing cases exhibited multiple mutations spanning different exons (3-7) which might be minor and have a limited role in functionally altering the receptor molecule. In normal functioning, the active region of EGFR in the intracellular domain is activated on binding of its ligand, EGF, to the extracellular domain that ensues in the transduction of further downstream signals. However alterations in the exons 3,4 and 6 as seen in persistent cases confer the EGFR protein constitutively active and also render the receptor un-susceptible to auto-inhibitory mechanisms. All GTD cases with mutations in the extracellular domain exhibited over-expression of EGFR-ICD and higher degree of protein phosphorylation and gene amplification. These results are suggestive that the amino acid substitution in the extracellular domain may modulate ligand binding and transmembrane signalling to the intracellular domain; thereby variant EGFR receptor may cause downstream activation of alternative signalling pathways, independent of ligand binding. The change in aminoacid sequence due to substitution/ insertion might also be the reason for the reduction in the EGF binding observed by us in persisting disease cases.

In an attempt to define the downstream signalling events of EGFR via the Akt/Ras pathways, the molar placenta revealed a significant increase in the expression of Akt protein in the persisting disease group ($p=0.005$), but no significant difference in expression of Ras protein. Significant association was obtained between the expression

of Akt and EGFR phosphorylation as well as gene amplification (all $p<0.001$). The elevated levels of Akt expression suggest an EGFR mediated Akt pathway to operate towards prolonged cell survival of trophoblastic cells, via anti-apoptotic events.

The rate of apoptosis in GTD, observed by laddering assay, was significantly less compared to normal placenta ($p=0.002$), and was of significant inverse association with EGFR gene amplification ($p=0.037$), expression of EGFR-ICD ($p=0.001$) and Akt ($p=0.003$); suggesting that the hypercellularity is due to defective apoptosis. The present observations indicate an EGFR/Akt mediated blockage of the apoptotic pathway in GTD.

The persistent disease group registered a significant decrease in the Bax expression ($p<0.001$), no significant difference in Bcl-2 expression and significantly decreased immunostaining of Caspase-3 ($p=0.031$). The association between down-regulation of Bax and caspase-3 with EGFR-ICD over-expression ($p= 0.042$ and $p=0.046$, respectively) and of Caspase-3 and Akt over-expression ($p=0.025$) was significant. Akt activation has been linked to the inhibition of Bax translocation to the mitochondria and Bax-induced mitochondrial permeabilisation. Several observations have also revealed that activation of Akt rescues cells from apoptosis by inhibiting Caspase-3 activity, and that Akt prevents membrane Phosphatidylserine exposure through inhibition of Caspase-9 and caspase-3. These results support the above observations and henceforth augment an Akt mediated blockage of the cell death pathway, via EGFR transactivation, in GTD.

Soluble isoforms of the EGFR is being explored as serum biomarkers with potential utility in the risk assessment, screening, and diagnosis of cancer. Values obtained from the GTD sera showed an increase in circulating EGFR ECD in 70.7 % of the cases ($p<0.001$) with 89.5% of the persisting disease cases showing high levels of sera ECD ($p=0.029$). The circulating levels of ECD showed significant correlation with the EGFR-ICD expression ($p=0.001$). The observations reported in this study indicate that serum sEGFR is a prognostic indicator of persisting disease and may have clinical utility as a new biomarker of persistent GTD. However additional clinical studies are warranted to evaluate whether serum sEGFR concentrations can be used to predict the prognosis of patients with GTD.