Molecular alterations in epidermal growth factor receptors as potential predictors of invasion in complete hydatidiform moles (CHM)

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Abstract. Molecular alterations in Epidermal growth factor receptor (EGFR) were investigated for the first time in molar placenta using protein expression, activation status, differential amplification status and mutational analysis. Invasive lesions showed upregulation of internal domain and downregulation of external domain with concomitantly high gene amplification and phosphorylation. Mutations distributed across different exons in non-invasive cases in contrast to single mutations restricted to exons 4 and 6 in invasive cases displayed a strong correlation to overexpression and phosphorylation status suggesting that higher copies of EGFR gene and mutations in exon 4&6 influence the invasive capacity of trophoblasts and can be used as a biomarker of invasion.

Keywords: EGFR, expression, mutations, CHM, invasion, biomarker

Abbreviations

CHM:	Complete Hydatidiform Moles
EGFR:	Epidermal Growth Factor Receptor
EGF:	Epidermal Growth Factor
HCG:	Human Chorionic Gonadotropin
EGFR-icd:	Epidermal Growth Factor internal domain
EGFR-ecd:	Epidermal Growth Factor external domain
PBS:	Phosphate Buffered Saline
BSA:	Bovine Serum Albumin
DAB:	Diamino Benzidine

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1. Introduction

Complete Hydatidiform Mole (CHM) is an abnormal conceptus characterized by significant hydropic enlargement and variable trophoblastic proliferation involving the chorionic villi. Orientals have a higher incidence of CHMs compared to the westerners. A high prevalence rate, similar to the highest rate in Asian countries, of this disease was reported in Kerala, India, with 12/1000 deliveries [1]. This disease carries social importance as it occurs in fertile females and is often a cause for marital disharmony.

Gestational trophoblastic moles are highly responsive to suction evacuation which forms the standard therapeutic mode. The serum beta HCG which is the sole marker available at present steadily drop to normal within 8–12 weeks after evacuation. However in 20% of the patients the serum HCG levels either rise or plateau or fluctuate. This is taken as an indication for malignancy and providing chemotherapy in the absence of an intervening pregnancy [2–4]. As of now, the capability of histopathology to accurately predict the

clinical behaviour of a hydatidiform mole and information about optimum management or prognostic factors is restricted. Invasive moles have the same histopathological characteristics as that of a non-invasive hydatidiform mole except for the infiltration of the trophoblasts into the myometrium and the necrotic changes associated with it. The characteristics of a hydatidiform mole that predisposes it to become a malignant tumour is not clear. Mounting evidence show that the molecular characteristics of the lesion may have a role to play in this process [5,6].

Epidermal Growth Factor Receptor (EGFR), anomalously expressed in multiple human epithelial neoplasms is reported to be associated with several key cellular processes such as, cell proliferation, survival, adhesion, migration and differentiation. Since CHM involves the proliferation of trophoblastic cells which are epithelial in origin, EGFR may have a pivotal role in its pathogenesis [7]. While over expression of EGFR has been associated with molar placenta, reports on its clinical significance have been conflicting [8–13]. In previous studies, a discrepancy was reported between EGFR expression in the non-invasive and invasive disease groups on using antibodies specific to the external and cytoplasmic domains. While a reduction was noticed in the expression of external domain of EGFR [12, 13], the cytoplasmic domain was overexpressed in most cases of the invasive disease group [11,13]. This was also substantiated by the observation of a reduced binding of EGF to the EGFR in invasive disease. These observations suggest a defect in the EGF binding domain of EGFR which could be due to truncation/mutation or deletion. An over expression of the cytoplasmic domain in invasive diseases due to amplification or mutation as suggested earlier [14,15] also cannot be ruled out. In this study, an attempt has been made to elucidate the precise mechanism for EGFR overexpression of internal domain and downregulation of external domain in CHM by analyzing the most commonly attributed reason, gene amplification or mutations in the gene. The activation status of the protein was also evaluated to assess its possible effect on the downstream events. This study is the first of its kind in literature wherein the alterations in the expression of the EGFR protein has been studied in CHM in relation to the clinical behaviour.

2. Materials and methods

2.1. Samples

A total of 107 fresh tissues of CHM, gestational age ranging from 6 to 36 weeks of amenorrhea collected

at the time of evacuation, were analyzed. The CHMs comprised of 69 cases that showed non-invasive lesions (NI) with serum beta HCG levels < 10 mIU/ml serum at 12 weeks following evacuation (non-invasive), 20 cases with serum beta HCG not reaching normal values of < 10 mIU/ml at 12 weeks of evacuation with fluctuating serum beta HCG levels (indecisive diagnosis group-ID) also termed persistent disease [2] and 18 that had signs of infiltration (invasive group-IN). The infiltration status was assessed by histological or ultrasonographic examination. The non-invasive and indecisive groups had no signs of infiltration or metastasis and two of the non-invasive disease had a rebound of high beta HCG values at 20 weeks of evacuation and was given chemotherapy for management. Fresh tissue was also obtained from 68 normal placentas of comparable gestational ages from therapeutic abortions. Informed consent was obtained from all individuals as per the directives of the Institutional Ethical committee of the Institution.

Classification of the lesions on the basis of gestational age showed that majority of the cases belonged to the first trimester of pregnancy < 12 weeks (98 cases), seven cases in the mid trimester 12–24 weeks of pregnancy and 2 cases in the final trimester ≥ 24 weeks of pregnancy. All cases belonging to the invasive group belonged to the gestational age group of < 12 weeks.

2.2. Immunohistochemistry for EGFR expression

Immunostaining was performed by the avidin-biotin method using antibodies against the intracellular domain (EGFR-icd) (Sigma Aldrich, USA) and the extracellular domain of the receptor (EGFR-ecd) (Santacruz, USA). Five-micrometer sections were de-paraffinised in xylene, re-hydrated in decreasing grades of ethanol, and quenched in 0.3% H₂O₂ in methanol for 30 min. The sections were then subjected to antigen retrieval in citrate buffer (10 mM), washed in PBS, and processed for the following steps. (a) Blocking with 3% BSA for 30 min, incubation with primary antibodies and subsequent incubation with biotinylated secondary antibody for 60 minutes at 37°C, (b) Washing in PBS, incubation with streptavidin horse radish peroxidase conjugate for 60 minutes at room temperature, (c) Incubating with diaminobenzidine (DAB) solution and H₂O₂ for 45 min. followed by washing in water for 10 min and staining with hematoxylin, dehydration with an ascending series of alcohol, clearing in xylene, air-drying and mounting. LSAB kit, DAKO Cytomation, Denmark was used for steps a–c. Negative control sections were

used for all samples from the same tissues. They were treated in parallel using normal blocking serum instead of primary antibody. To quantify the extent of EGFR expression, a grading system was used ranging from 1 to 4 based upon the intensity of the staining.

2.3. Western blot analysis for EGFR expression

Frozen tissue was homogenised and lysed in 20 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA (pH 8.0) 10% glycerol, and 1% Triton-X₁₀₀, containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 2 µg/ml aprotinin, and 5 µg/ml leupeptin) for protein extraction. Protein extracts prepared from the frozen tissue (200 µg) was loaded on a 10% SDS-PAGE gel, electrophoresed and transferred onto a polyvinylidene-fluoride transfer membrane (Millipore). After 2 hours blocking in 5% non fat dry milk, the membrane was incubated overnight with EGFR primary antibodies (EGFR-icd (Sigma Aldrich, USA) or ecd (Santa Cruz Biotechnology, Santa Cruz, CA) and washed in TBS-Tween 20. The membrane was incubated again for 1 hr with horse-radish peroxidase conjugated secondary antibody (Bangalore Genei, India), washed and the reactions were visualized by developing with BCIP/NBT (BCIP/NBT kit, Bangalore Genei, India).

2.4. Protein lysate ELISA test for evaluation of activated EGFR

The phosphorylation status of the EGFR protein was assessed using ELISA kit from Bendermed systems, (Bender MedSystem®, Bender MedSystems GmbH, Vienna, Austria), to compare the levels of phosphorylated (activated) EGFR in molar and normal placenta. Tissue protein lysates were diluted in the ELISA kit diluent and analysed as per supplier's protocol. The samples were tested in duplicate along with standards. The concentration of the phosphorylated EGFR protein was determined in fmols from standard curve. The mean value + 1SD of the normal placental samples was taken as normal. Any GTD sample that was above the normal value (mean + 1SD) was considered to have increased levels of phosphorylated EGF receptors.

2.5. Differential PCR to evaluate amplification status of EGFR

DNA was extracted from frozen tissue samples and diluted to (200 ng/5 µl). The primers used were

as follows. EGFR: 5'-AGCCATGCCGCATTAGCTC-3' (sense primer) and 5'-AAGGAATGCAACT TCC-CAA -3' (antisense primer); these primers amplify a 110 bp genomic fragment corresponding to bases 3901–4010 in the cytoplasmic domain of the EGFR molecule. β -globin: 5'-TGACTCCTGA GGAAGAA GTCTGC-3' (sense primer) and 5'-TCACCACCAAC TTCATCACGT-3' (antisense primer); these primers amplify a 70bp fragment from the β -globin gene (cDNA bases 164–186).

Each 25 µl reaction mixture contained 2.5 µl Taq buffer (Biogene, India) 2.5 µl 0.01% gelatine, 2.5 µl dNTP Mix (Bangalore Genei, India) (2000 µM) 2.5 µl of each primer (Bangalore Genei, India), 0.2 µl Taq polymerase enzyme (Biogene, India), 2.3 µl Sterile Distilled water, and 5 µl of the DNA template. Two controls that contained all the reagents but no target DNA were included with each batch. Each reaction mixture was placed at 92°C for two minutes and then subjected to first 5 amplification cycles; each cycle being 30 seconds at 97°C, 1 minute at 53°C, and 1 minute at 72°C; and then to another 30 cycles; each cycle being 1 minute at 95°C, 1 minute at 53°C, and 1 minute at 72°C. This was followed by a final extension at 72°C for ten minutes.

Amplification products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and visualised under ultraviolet illumination. The gel images were captured using the Gel-Doc 1000 photo documentation system (Bio-Rad, Philadelphia, USA) and were quantitatively analysed using Quantity One software. Samples with an EGFR band intensity greater than 200% in comparison with the β -globin band intensity, was considered as amplified for the EGFR gene.

2.6. Evaluation of molecular alterations

For mutational analysis of the EGFR, extracellular ligand binding domain coding sequence, exon 2, 3, 4 (domain I), and 5,6 and 7 (domain II) of the EGFR were amplified with six pairs of primers [16], specific to the flanking sequences of individual exon. Custom oligonucleotide primers purchased from Mssrs. Bangalore Genie, India were used.

Each 15 µl reaction mixture contained 1 µl Taq buffer (Biogene, India), 1.6 µl 50% glycerol, 1 µl dNTP Mix (Bangalore Genei, India) (2000 µM), 2 µl each of forward and reverse primers, 0.1 µl Taq polymerase enzyme (Biogene, India), 0.8 µl sterile distilled water, and 1.5 µl of DNA template (made up to 5ng/5 µl). PCR

cycles were carried out as follows. 95°C for 5 minutes, cycles 1–41 at 94°C for 30 seconds, 60°C for 45 seconds and 72°C for 45 seconds, and a final extension after the last cycle at 72°C for 10 minutes. The PCR amplicons were outsourced for direct sequencing (Bangalore Genie, Bangalore, India). Forward and reverse sequencing reactions were done with the same primers for PCR amplification; sequencing reactions were electrophoresed on an ABI3700 genetic analyzer. Sequence variations were determined with the EGFR reference sequence (NM_005228.3, NCBI), using Sequence Scanner software (Applied Biosystems, USA) and BLAST services (DDBJ). The status of tumours was reported only if complete readable sequence was obtained for all exons. All cases were confirmed by PCR amplification of an independent DNA isolate.

2.7. Statistical analysis

The statistical significance of data was evaluated using Chi-square tests. The level of significance was assigned at $P < 0.05$. All analyses were performed using SPSS version PASW Statistics 18.

3. Results

3.1. Expression of EGF receptor

Immunohistochemically, staining with anti-EGFR antibodies specific to the intracellular domain (icd) and to the extra-cellular domain (ecd) was seen both in the cyto- and syncytio-trophoblasts of normal placenta and molar placenta. EGFR-positive cells demonstrated a partly membranous, partly cytoplasmic staining pattern. Since the normal placenta of corresponding gestational age also showed negative to moderate staining, the negative and mild staining patterns were grouped as negative and moderate and intense were grouped as positive for Chi-square analysis. In comparison with normal placenta, EGFR-icd showed increased expression in 76.6% of CHM lesions with a mean staining index of 3.72 ± 0.08 versus 2.82 ± 0.007 in normal placenta ($p < 0.001$), when taken as a whole. The EGFR-icd protein over expression was seen in 59.4% of the non-invasive lesions (mean staining index 3.31 ± 0.10 , $p = 0.010$), 68.6% of the indecisive diagnosis group (mean staining index 3.62 ± 0.18) and 94.1% of the cases in the invasive group (mean staining index 3.99 ± 0.01) (Table 1).

Fig. 1. Western blot representing the protein expression of EGFR-ecd and EGFR-icd in normal placenta and CHM. NI – Non-Invasive CHM, ID – Indecisive CHM, IN- Invasive CHM, N.PL- Normal Placenta.

The expression of EGFR-ecd ranged from mild to intense intensity in CHM. A reduced level of expression was observed in 94.3% of invasive group (mean staining index 2.24 ± 0.008) when compared to that in the non-invasive group (mean staining index 2.70 ± 0.006) and the normal placenta (mean staining index 2.75 ± 0.007 , p -value 0.009) (Table 1).

Chi-square analysis indicated a higher risk among patients with over-expression of EGFR-icd to have CHM to be 7.251 (95% CI 3.590–14.644) when compared to those with normal expression (p -value < 0.001). The CHM patients whose lesions over expressed EGFR-icd also showed a higher RR of 12.056 to develop invasive disease (95% CI = 3.825–38.000, p -value < 0.001) in comparison to non-invasive disease (RR = 3.185, 95% CI = 1.564–6.487, p -value 0.001).

Expression of EGFR-icd was observed to be higher in CHM in all the gestational age groups when compared to that in placenta. In normal placenta, the highest expression was observed in the first trimester with a diminishing expression in the last trimester of pregnancy. This phasic pattern was absent in the CHM cases for EGFR-icd.

The immunohistochemical observations were confirmed by western blot profiles of the same samples (Fig. 1). Increased band intensity was noted in cases of invasive CHM when compared to non-invasive cases, identical to the IHC results of the same samples.

3.2. Activation status of EGF receptor

Assessment of the phosphorylation status of EGFR protein by ELISA revealed 56 percent of the CHM cases to exhibit significantly higher levels of phosphorylation ($p < 0.001$). The increase in percentage of invasive cases and indecisive cases showing high levels of EGFR phosphorylation was highly significant ($p < 0.001$ and

Table 1
Molecular alterations observed in Gestational Trophoblastic diseases

Parameter	Placenta	Gestational trophoblastic diseases		
		Non-invasive lesions	Indecisive lesions	Invasive lesions
<i>Number studied</i>	68	69	20	18
<i>EGFR Expression</i>				
Internal domain (EGFR-icd)	2.82 ± 0.007	3.31 ± 0.10*	3.62 ± 0.18*#	3.99 ± 0.01*#
External domain (EGFR-ecd)	2.75 ± 0.007	2.70 ± 0.006	2.64 ± 0.008	2.24 ± 0.008*#
<i>EGFR phosphorylation status</i>				
Normal	68 (100%)	35 (50.7%)*	8 (40.00%)*	6 (33.3%)*#
High	0	34 (49.2%)*	12 (60.00%)*#	17 (94.4%)*#
<i>Positive EGFR gene Amplification</i>	0%	7 (29.2%)*	7 (63.4%)*#	15 (83.3%)*#
<i>EGFR Mutations (12 in each group)</i>				
No mutations	100%	0%	50%	–
Substitutions	0%	50%*	–	100%*#
Substitutions & insertions	0%	50%*	50%*	–

* Alteration significant ($X^2p < 0.001$) when compared to normal placenta.

Alteration significant ($X^2p < 0.001$) when compared to non-invasive lesions.

$p < 0.001$, respectively), when compared to that in the non-invasive cases (Table 1).

The phosphorylation levels of EGFR which indicates the active status of the protein also correlated directly with the expression levels of the receptor molecule ($p = 0.035$). Compared to the cases of CHM expressing low and normal phosphorylation levels of EGFR, the relative risk of those with high levels of phosphorylated EGFR towards invasive disease was found to be more than 31 fold (95% CI = 0.003–0.300, p -value < 0.001) while the same to predict non-invasive disease was only 19 times (95% CI = 0.006–0.458, p -value < 0.001).

3.3. EGFR gene amplification

Multiplex PCR of CHM specimens revealed preferential amplification of the EGFR fragments when compared to β -globin reference. In all, 45% of the molar placenta evidenced significantly increased EGFR band intensity over the reference level, even though the same amount of DNA was subjected to amplification for both EGFR and β -globulin ($p < 0.001$) (Fig. 2) with 29.0 percent of the non-invasive and 83.3% of the invasive disease cases demonstrating an amplified EGFR gene copy number ($p < 0.001$).

The molar placenta with EGFR gene amplification also exhibited elevated EGFR expression on immunohistochemical analysis ($p = 0.008$), as well as high levels of EGFR phosphorylation ($p < 0.001$).

On Chi-square analysis, the risk of patients with EGFR gene amplification relative to patients without gene amplification to develop CHM was 16 fold with a 95% CI of 0.07–0.500 and a p -value of < 0.001 while the risk to develop invasive disease was found to be 44

times (95%CI = 0.002–0.220, p -value < 0.001); and that to develop non-invasive disease was 8 fold (95% CI = 0.014–1.088, p -value > 0.05).

3.4. Molecular alterations in the EGFR

Randomly selected 10 cases of normal placenta and 36 cases of EGFR over expressing CHMs comprising of 12 non-invasive cases, 12 indecisive cases and 12 invasive cases were subjected to sequencing of the PCR products of exons 2–7 which included the EGF binding domain of the EGFR molecule.

A significantly higher number of CHM cases showed mutations in the extracellular domain of EGFR ($p < 0.001$). No genetic alteration was observed in exon 2 of EGFR gene in any of the samples studied. Exon 4 registered the highest incidence of genetic alterations including polymorphism and mutations in CHM cases (83.3%) along with concomitant or single mutations in other exons, whereas the only genetic alteration (polymorphism) found in placenta was exon 4 polymorphism in all cases studied. All non-invasive cases displayed either polymorphism or mutation in exon 4 concomitant with mutations in exon 7 in all cases and exon 5 in 50% cases. The other 50% had mutations spread over exons 3–6 along with the mutations in exon 4 and 7. The invasive disease showed gene alterations in exon 4 in all cases concomitant with alterations in exon 6 in 52% cases. No alteration was observed in any of the other exons in these cases. In the indecisive cases, 50% of the cases had only polymorphism noted in exon 4 while 33.3% (4/12) had alterations in exon 4 concomitant with mutations in exon 3 and 16.6% (2/12) had alterations in exon 4 concomitant with mutations in

Table 2
Functional types of EGFR mutations involved in normal placenta and GTD

	No mutation	Silent	Mis-sense	Mis-sense & Non-sense	Silent & Mis-sense
Placenta	10 (100%)	–	–	–	–
GTD	6 (17%)	12 (33%)	6 (17%)	6 (17%)	6 (17%)
Non-invasive	–	–	6 (50%)	6 (50%)	–
Indecisive	6 (50%)	–	–	–	6 (50%)
Invasive	–	12 (100%)	–	–	–

Fig. 2. Gel picture representing Differential PCR products showing the amplification status of EGFR gene in normal placenta and CHM. MWM- Molecular weight marker, NI – Non-Invasive CHM, ID – Indecisive CHM, IN- Invasive CHM, N.PL – Normal placenta (early and mid pregnancy). Arrows indicate cases exhibiting Gene amplification.

exon 6 (Fig. 3). Single mutations were observed only in the invasive and indecisive lesions. No alterations were observed in exon 5 in these cases. The percentage mutations noticed in the CHM group was significant as no mutations were observed in the placenta.

Sequencing results showed the gene alterations observed to be either polymorphisms or substitution mutations or substitution and insertion mutations. Tables 1 and 2 and Fig. 3 give the details of the molecular and functional types of EGFR mutations in placenta and CHM lesions. The alterations observed in infiltrating cases were all substitution mutations while the indecisive cases had both substitution and insertion mutations. Nonsense mutations were found only in spontaneously regressing cases.

It was interesting to note that all the cases with mutations, both silent and missense, over expressed EGFR-icd. Significant correlation was obtained between the presence of mutation and high levels of EGFR protein phosphorylation ($\chi^2 = 0.035$) as also with presence of EGFR amplification ($\chi^2 = 0.014$).

4. Discussion

This study was based on the earlier observations of conflicting observations of expression of EGFR in non-invasive and invasive cases of CHM. Many types of epithelial malignancies are reported to express increased levels of EGFR on the cell membrane, as determined mainly by immunohistochemistry (IHC) showing correlation with disease progression, poor survival, poor

response to therapy [13,16–18], tumour aggressiveness and development of resistance to cytotoxic agents [19]. A similar situation is reported in vesicular moles where over expression of EGFR correlated well with disease progression when assessed using antibodies to the internal domain [13]. Ligand binding assay, which measured the external ligand-binding domain of EGFR, and binding of antibody to the external domain, however, showed an inverse relationship with aggressiveness [12, 13] suggesting the presence of receptors with either mutations or deletions of the external domain similar to that reported in glioblastomas [20,21]. The immunohistochemical and immunoblotting findings in the present study concur with previous reports, indicating an over expression of EGFR with regard to its intracellular domain and a down regulation with respect to its extracellular domain in invasive trophoblastic disease. Activation of EGFR has been shown to enhance processes responsible for tumour growth and progression, including the promotion of proliferation, angiogenesis, and invasion/metastasis, and inhibition of apoptosis [22,23]. Such over expression produces intense signal generation and activation of downstream signalling pathways, resulting in cells that have more aggressive growth and invasiveness characteristics. This study has observed relative risk levels of 12.05 for EGFR-icd over expression for development of invasive CHM, suggesting that this receptor may play a role in proliferation and aggressive progression of gestational trophoblastic diseases. Further, the findings also suggest that this receptor may also constitute a potential therapeutic target in persistent CHMs as indicated by Fulop et al. [24].

Fig. 3. Representative electropherograms of genetic alterations in exons 2–7 of EGFR in GTD when compared to normal placenta. A–D: Alterations in exon4. Polymorphism in exon 4 (C 474 T; Asn 58 Asn)– Normal placenta and some Indecisive GTD (A), GTD (B); Substitution Mutation (G 528 A; Met 176 Ile) – Normal placenta (C), Invasive and Indecisive GTD (D). E–J: Alterations in Exon 3. Substitution Mutation (C276 A/C277 G; Ala 92 Ala/ Leu 93 Val)) – Normal placenta (E), Indecisive GTD (F); Substitution Mutation (T 367 A/T 367 G; Ser 123 Thr/ Ser 123/Ala)) – Normal placenta (G), Indecisive GTD (H); Insertion mutation (CG 339–340 CAG; Glu (114) Asn Ser Tyr/Arg. (114) Lys Phe Leu.)) – Normal placenta (I), Indecisive GTD (J). K–L: Alterations in Exon 5. Substitution Mutation (A 623 T / G 624 C; Gln 208 Lys/ Gln 208 His) – Normal placenta (K), Non-invasive GTD (J). M–N: Alterations in Exon 6. Substitution Mutation (C 680 A / C 680 T / C 681 G; Ser 221 Tyr/ Ser 221 Phe/ Ser 222/Ser) – Normal placenta(M), Invasive GTD (N). O–P: Alterations in Exon 7. Substitution Mutation (A 877 T / A 878G / A 878 C; Lys 293 stop/ Lys 293 Arg/ Lys 293 Thr) – Normal placenta(K), Non-invasive GTD (J).

Increasing levels of wild-type EGFR results in constitutive tyrosine phosphorylation of the receptor, presumably secondary to dimerisation in the absence of exogenous ligand [25]. Trafficking of the EGFR is also altered in over expressing cells with decreased internalisation, increased recycling and prolonged membrane persistence of the activated receptor [26]. In addition, increased expression of the receptors overwhelms mechanisms for dephosphorylation of receptors. All these changes favour a state of unattenuated and persistent signalling [25].

The present observation of high levels of phosphorylated EGFR protein in CHM and positive correlation with aggressiveness of the disease is supported

by previous reports on active status of the receptor in various tumours, further reiterating the role of active EGFR resulting in abnormal trophoblast cell accumulation in this disease. Phosphorylated tyrosine residues in the EGFR is reported to activate the Ras/Raf/mitogen-activated protein kinase (MAPK) signalling cascade, which, in turn, influences cell proliferation, migration, and differentiation [27,28] which also results in inhibition of apoptosis [27]. These studies lead us to infer that the over expressed EGFR protein is constitutively active conferring persistent signals for proliferation/cell survival in CHM, thereby contributing towards the aggressive phenotype or persistence of molar tissue. This is the first report on the activation status of

epidermal growth factor receptor protein in gestational trophoblastic disease.

Mechanisms that mediate EGFR over expression include gene amplification, truncation of the carboxyl terminus, transcriptional activation, and posttranslational modifications [29,30]. Over expression of the EGFR is commonly caused by EGFR gene amplification and is sometimes associated with expression of a variant EGFR (de2-7 EGFR or EGFRvIII) bearing an internal deletion in its extracellular domain [31]. The present gene amplification studies showed a significantly higher number of patients with CHM to have amplification of the EGFR gene which was also reflected in over expression of the protein. The frequency of amplification in this series, 45%, is consistent with that reported by other groups [14,32]. The very high relative risk (44 fold) of developing invasive disease for samples with gene amplification compared to those that did not show any evident amplification of the EGFR gene further implies the role of this phenomenon in piloting trophoblast cells to adopt an aggressive phenotype in CHM. The relative risk estimate of EGFR gene amplification along with the high levels of EGFR protein phosphorylation (44 fold and 31 fold respectively), if evaluated together, should be able to identify patients with risk of developing invasive disease as early as initial diagnosis, the potential of which needs to be warranted in a larger study. A high concordance between gene amplification, abnormality of EGFR gene and protein over expression have been reported in various other tumours [26,33–36]. The significant correlation with increased EGFR expression and presence of EGFR gene amplification suggest that gene amplification is primarily responsible for EGFR over expression in CHM and also suggest that the over-expression of this receptor protein in CHM may be a result of disturbed transcriptional mechanisms ensuing from higher copies of the EGFR gene. Significantly high percentage of samples positive for EGFR amplification among the CHM cases, especially, invasive cases suggest that EGFR amplification confers a survival advantage to pathological trophoblasts. Experimental evidence also suggest EGFR amplification to result in a less favourable prognosis [33,35]. However, similar to previous reports, some of the over expressing cases of GTD have also registered negative for presence of gene amplification suggesting that there are multiple mechanisms involved in EGFR protein over expression [37] and alterations of the gene secondary to amplification may be important toward the oncogenic effect of EGFR [38,39].

Exon 4 polymorphism seen in normal placenta and majority of CHM does not confer any alteration to

the EGF receptor molecule as it is of a silent nature. This polymorphism has been registered in the EGFR polymorphism database (ref ID rs2072454, www.cityofhope.org/cmdl/egfr_db) and consists of substitution of cytosine with thymine at base pair 474 coding for the same amino acid (Asn), thereby rendering this polymorphism silent.

The most common mutation resulting in an EGFR with a constitutively active tyrosine kinase (EGFRvII), has been shown to confer enhanced tumourigenicity *in vivo* and correlate with poor prognosis [40]. The present study revealed that all the cases of mutations observed were invariably in samples exhibiting presence of EGFR gene amplification. Similar reports have been proposed earlier suggesting that EGFR mutations are limited to tumours with EGFR amplification and include single nucleotide substitutions [35] and that EGFR amplification acts as a prelude to and a mechanism for inducing alteration of the gene. Early reports on EGFR mutations in other cancers have identified presence of missense mutations in tumours with EGFR amplification [32], which is in concordance with the presence of these types of mutations noted in the present study. Tam et al. [41] has reported 53% amino acid substitutions, 43% in-frame deletions, and 4% insertions in lung cancers. The EGFR mutation database (www.cityofhope.org/cmdl/egfr_db) also registers base substitutions to account for majority of the reported EGFR gene mutations (51.6%). The findings in this study also report greater incidence of substitutions (83.3% in CHMs, 100% in invasive cases) compared to insertions. The alteration in the amino acid sequence of the external EGF binding domain of EGFR would also explain our observation of reduced binding of EGF in the invasive cases of CHM.

Recently, several studies have identified somatic mutations in the tyrosine kinase domain of the EGFR gene [42], as activating mutations involved in tumourigenesis. The extracellular domain of EGFR dimerises by virtue of homophilic interactions involving a specific loop projecting from each of the domain II (encoded by exons 5–7) of the two adjoining extracellular domains. The point mutations observed in this study involve domain I and domain II. It is evident from the EGFR dimerisation kinetics that these mutations may play a part in the active signalling of EGFR molecule, maybe in a mode devoid of need for EGF ligand binding. The crystal structures are consistent with the “receptor-mediated” mechanism for dimerisation [43], and mutations obtained in domain I may affect EGF ligand activation of the receptor molecule

since conformational changes might ensue following mutations which might be conferring an escape mechanism from the auto-inhibitory effects of ligand binding which could possibly explain the significant correlation of presence of mutations with the active status of EGFR in CHM. These observations further imply that the EGFR mutations detected in this study may be functional alterations similar to that indicated in a study by Lee et al. [44] with overall similar results. Most of the non-invasive cases of CHM in our study harboured mutations that involved random exons while the invasive cases recorded mostly single mutations in exon 6 coupled with single mutations in exon 4. Mutations in exon 7 and 5 were seen only in the spontaneously regressing cases and this alteration might be offering them the propensity to escape ligand binding but not offering the aggressiveness. Though it might appear that spontaneously regressing group harbour a greater variety of mutations than invasive disease type, they may be minor as they are scattered over various exons and only those in the invasive disease cases may prove sufficient for receptor activation. The indecisive group of CHM showed mutations in exon 6 in 16.6% cases along with mutations in exon 4 with no mutations in any other exons resembling the invasive cases suggesting that these may have harboured invasive trophoblasts which might have escaped diagnosis by the ultrasonography or histopathology examinations. The finding of mutations in exon 3 in 40% of the cases in these cases is surprising as they are neither seen in the non-invasive nor the invasive cases. These results thus represent mutations in exon 6 and 4 in invasive cases, and wider distribution across different exons (3,4,5,6 and 7) in non-invasive cases. Reports have shown domain I (encoded by exons 2–4) alterations to confer EGFR molecules constitutively active [45]. Alterations in EGFR domain II (encoded by exons 5–7) affects the positioning of the EGFR molecule assuming altered conformations eventually making the receptor capable of evading the auto-inhibitory mechanisms [46]. Hence it is plausible to suggest that the alterations we have noticed in exons 4 and 6 in case of the invasive disease might be responsible for the reduced binding of EGF [13] and the reduced binding of the antibody seen in this study. It also appears that the combined mutations in exon 4 and exon 6 apart from conferring a constitutively active status to the EGFR molecule, also provides a higher invasive capacity to the trophoblasts.

5. Conclusions

The present study reports the potential of molecular alterations leading to the over expression of internal domain and reduced expression of the external domain of EGFR proteins as biomarkers in invasive lesions of CHM. The current methods have limited capacity to detect all invasive cases of CHM and hence this study is important. Further, for differential expression studies, most studies have used antibodies against the internal domain of EGFR which is overexpressed both in the invasive and non-invasive lesions and is not able to discriminate between the two. The reduced expression of the external domain of EGFR concomitant with reduced ligand binding observed in earlier studies appears to be associated with the invasive lesions. The molecular alterations related to overexpression of the gene or the activation status of the protein has not been investigated in literature in CHM and this study shows that the overexpression of EGFR in invasive lesions is closely related to gene amplification and to increased phosphorylation. This study also reveals the presence of mutations in the external domain which correlates with its antibody responsiveness. This is the first study where the activation status of EGFR and mutation of the external domain of EGFR has been demonstrated in CHM and suggests that concomitant mutations in Exon 4 and 6 hold potential as markers for invasiveness and CHM patients requiring chemotherapy can be identified right at the time of initial investigations.

Conflict of interest statement

The authors have no conflict of interest of any type.

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