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ORIGINAL PAPER

Human *EGFR*, a candidate gene for the Silver-Russell syndrome, is biallelically expressed in a wide range of fetal tissues

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Maternal uniparental disomy of chromosome 7 (mUPD7) has been reported in around 10% of cases of Silver-Russell syndrome (SRS). This suggests that at least one gene on chromosome 7 is imprinted and involved in the pathogenesis of this condition. One candidate is epidermal growth factor receptor (*EGFR*) which maps to chromosome 7p12, a region homologous to an imprinted region on mouse chromosome 11. Using a restriction fragment length polymorphism, biallelic expression of *EGFR* was found in a range of normal human fetal tissues. Expression was also demonstrated in fibroblasts and lymphoblasts from SRS patients with mUPD7. Thus no evidence that *EGFR* is imprinted was found, making its involvement in SRS unlikely. However, *EGFR* was shown to be widely expressed in the human fetus, evidence that this gene plays an important role in early development.

Keywords: epidermal growth factor receptor; Silver-Russell syndrome; maternal uniparental disomy; imprinting; fetal expression

Introduction

Maternal uniparental disomy for chromosome 7 (mUPD7) has been described in several patients with intrauterine growth retardation (IUGR).[1–3] More recently, approximately 10% of cases of Silver-Russell syndrome (SRS) have also been found to be associated with mUPD7.[4,5] SRS is characterised by intrauterine and postnatal growth retardation with relative sparing of cranial growth, triangular facies and downturned corners of the mouth. In a large proportion fifth finger clinodactyly and facial, limb or truncal asymmetry is also present. In five SRS patients with mUPD7 studied by our group, no consistently isodisomic areas have been found (unpublished observation). This makes exposure of a recessive gene an unlikely explanation for the phenotype associated with mUPD7, suggesting instead that one or more gene(s) on chromosome 7 are imprinted and play a role in the pathogenesis of SRS and IUGR in some cases.

Likely imprinted candidate genes can be sought by comparison of mouse and human linkage maps. Several

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regions of human chromosome 7 have been shown to be homologous with imprinted regions in the mouse, including proximal chromosome 11.[6] This region, homologous with human chromosome 7p12-13, leads to mice with prenatal growth failure when maternally disomic and to prenatal overgrowth when paternally disomic. The human epidermal growth factor receptor (*EGFR*) gene maps to 7p12 within this region and has been proposed as a candidate gene for SRS.[4,5]

EGFR is a 170-kDa transmembrane glycoprotein which mediates the effects of epidermal growth factor (EGF) and transforming growth factor-α(TGFα).[7] Several observations suggest this gene has an important role in intrauterine growth and therefore deserves further investigation. *EGFR* is expressed in a wide range of murine embryonic and fetal tissues[8] from as early as the preimplantation stage.[9] Demonstration of *EGFR* expression in the human fetus has, until now, been limited to a narrower range of tissues but has been described in placenta,[10] ovary and uterus,[11] lung,[12] keratinocyte, endothelial and skeletal muscle cells.[13] These results suggest that one or more ligand(s) for EGFR are widely involved in embryonic and fetal development. Other studies provide evidence for a specific role in growth. Rat embryos explanted on day 9.5 and cultured in the presence of a specific EGFR tyrosine kinase inhibitor, show dose dependent growth retardation.[14] Furthermore, in the human, IUGR is associated with a reduction in placental EGFR.[15>]

Both natural missense mutations and artificial 'knockouts' of murine *Egfr* have been described.[16,17] The phenotype of homozygote mutants varies depending on genetic background but includes IUGR in addition to abnormalities of skin, hair and eyes. Significantly, heterozygotes are normal, whichever the transmitting parent, suggesting that the gene is not imprinted in the mouse. Differential parental gene expression of murine *Egfr* has also been investigated in whole embryos with maternal/paternal disomy of proximal chromosome 11.[18] No evidence of imprinting was found at a number of different stages. However, there is a growing number of reports of tissue,[19] developmental stage[20] and species-specific[21] imprinting of genes. These findings make it important to study the allele-specific expression pattern of *EGFR* in a number of human fetal tissues at different gestational ages to confidently rule out its involvement in the mUPD7 phenotype. We have therefore investigated parent of origin expression of *EGFR* in normal first and second trimester fetuses and also its expression in SRS patients both with and without mUPD7. During this study we observed *EGFR* expression in a much wider range of human fetal tissues than has previously been reported.

Materials and Methods

Patient Sample

Clinical details of the SRS patients (HW, and GB) with mUPD7 have been described by Preece *et al*.[5] Their ages were 6.8 and 3.3 years. The three SRS patients (JC, MA and TL) without mUPD7 in whom *EGFR* expression was studied were 36.5, 6.1 and 9.4 years old respectively.

Isolation of DNA and RNA from Tissues

Normal fetal tissues for investigation of *EGFR* expression were obtained from the MRC Tissue Bank at Hammersmith Hospital (L Wong). Further samples for investigation of *EGFR* imprinting status were collected from pregnancies terminated at Queen Charlotte's and Chelsea Hospital. Specimens were stored at –80°C until use. For investigation of imprinting status, 5 ml of paired maternal blood was also collected and stored at –20°C. Local ethics approval for collection of maternal and fetal samples was granted by the Research Ethics Committee of the Royal Postgraduate Medical School (96/4955). Genomic DNA was extracted from fetal tissue and maternal blood as described by Kunkel *et al*.[22] RNA was isolated from fetal tissues using a standard guanidinium technique.[23]

Expression Analysis

RNA samples (1 μg) were denatured at 70°C for 5 min and cooled to 37°C. Reverse transcription was carried out using random hexanucleotide primers (0.2 μg), 1× reverse transcriptase buffer, 10 mM dithiothreitol, 1 mM each dNTP, 40 units M-MLV reverse transcriptase (Gibco BRL) and 1 unit RNase inhibitor (Pharmacia Biotech). These were added to a final volume of 20 μl, incubated at 37°C for 60 min and the reaction then terminated by heating to 90°C for 5 min. PCR using primers for *GAPDH* confirmed the presence of cDNA. Primers EGFR-F and -R, used to study *EGFR* expression, were identical to the outer EGFR primers described by Chia *et al*.[24] These primers have minimal homology with other *EGFR*-related receptors (e.g. c-erb B2 or tyrosine consensus sites) and span an intron, allowing detection of contaminating genomic DNA. 1/20 volume of the RT template was used for each 25 μl PCR reaction containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 125 ng of each primer and 1 unit of Taq polymerase (Biotaq). Reaction conditions were 4 min at 94°C, followed by 30 cycles denaturation for 1 min at 94°C, annealing for 1 min at 61°C and extension for 1 min at 72°C, and a final extension for 8 min at 72°C. Products were run out on 1% ethidium bromide stained agarose gels. Expression was studied in 8 different tissues (placenta, liver, brain, lung, gut, heart, skin and kidney). Three sets of tissues from both 10–12 and 16–18 week stages were analysed. Each set of tissues was derived from several different fetuses. Primers EGFR-F and -R were also used to detect expression of *EGFR* in fibroblasts and lymphoblasts from SRS patients.

Investigation of Imprinting Status

Primer sequences (A1/A2, B1/B2) spanning the polymorphic *BstN*I restriction site were identical to those described by

Moriai *et al*.[25] For primer set A1/A2 the following conditions were used: 4 min at 94°C followed by 35 cycles denaturation for 1 min at 94°C, annealing for 1 min at 59°C and extension for 1 min at 72°C, and a final extension for 8 min at 72°C. For primer set B1/B2 25 cycles were used but conditions were otherwise identical.

Primers B1/B2, which do not span an intron, were used to screen genomic DNA extracted from maternal and fetal tissues. 14 μl of the PCR product was digested with *BstN*I (New England Biolabs) at 65°C for 90 min. Alleles were separated on 4% agarose ethidium bromide stained gels. RT-PCR was then performed on RNA isolated from the tissues of fetuses found to be heterozygous, using primers A1/A2. These primers span an intron and thus allow genomic DNA to be distinguished from expressed cDNA sequences. The products were digested and visualised in the same way.

Cell Culture

Fibroblast cell lines were generated from punch skin biopsies. Cells were grown in Ham's F-10 Medium (Imperial) supplemented with 30% fetal bovine serum (Globepharm) and 2 mM L-glutamine under the same conditions. Fibroblast cells were harvested while still subconfluent by removal of growth media, addition of 1 ml Trypsin-Versine (0.125% trypsin, 0.125% EDTA in PBS) and incubation for 5 min at 30°C. Peripheral blood lymphocytes were used to create EBV immortalised cell lines at the ECACC (European Collection of Cell Lines), Centre for Applied Microbiology, CAMR, Porton Down, Salisbury, UK. Cell lines were then grown in RPMI 1640 culture medium (Gibco BRL) supplemented with 10% fetal calf serum (Sigma) and 2 mM L-glutamine in 5% CO_2 atmosphere at 37°C. Lymphoblast and fibroblast cell pellets were obtained by spinning cells in suspension at 1000 rpm for 5 min; mRNA was isolated from these pellets using the same guanidinium isothiocyanate technique as used on fetal tissues.

Results

Expression of EGFR *in First and Second Trimester Fetal Tissues*

RNA was extracted from placenta, skin, brain, liver, heart, intestine, kidney and lung. Normal fetal tissues from both first (10–12 week gestation) and second (16–18 week gestation) trimester were used. Expression of *EGFR* mRNA was investigated by means of reverse transcriptase PCR (RT-PCR). PCR products of the expected size (208 bp) were seen in all tissues analysed from both gestational stages (Figures 1a and b). Sequence analysis of this 208 bp product confirmed that it matched the published sequence for *EGFR* in GenBank database (from nucleotides 3207 to 3414, accession no. X00588) (results not shown). PCR using genomic DNA as template gave two products approximately 750 bp and 1.3 kb in length. Thus products due to contamination with genomic DNA could easily be distinguished.

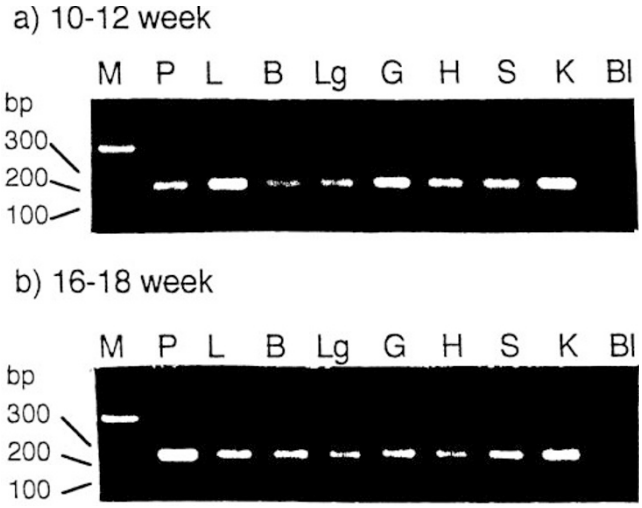

Figure 1 *Gel electrophoresis of RT-PCR products, using primers EGFR-F and -R, showing widespread* EGFR *mRNA expression in (***a***) 10–12 week gestation tissues and (***b***) 16–18 week gestation tissues. Tissue types are: placenta (P), liver (L), brain (B), lung (Lg), gut (G), heart (H), skin (S) and kidney (K). M: 100 bp marker; Bl: blank (no template)*

Expression was analysed in two further sets of the same tissues (see methods) from both first and second trimester stages. In each, the same ubiquitous pattern was seen at both gestations. Of the human fetal tissues included in this study, *EGFR* expression had only previously been described in skin, lung and placenta. In addition, we have demonstrated transcripts in liver, brain, gut, heart and kidney. Since the PCR cycle number used was outside the linear range no conclusions can be drawn about the relative level of expression in the various tissues at different stages.

Investigation of Imprinting Status

A polymorphism within the coding region of the *EGFR* gene was previously described by Moriai *et al*[25] (Figure 2). The observed heterozygosity in 20 unrelated individuals was found by us to be 0.38. This transition, which alters a *BstN*I restriction site, was used to investigate parent of origin expression of the alleles. Genomic DNA was prepared from fetal tissue from 9 to 18 weeks gestation and matched maternal blood samples. Primers B1 and B2 were then used to amplify the region spanning the polymorphism. Out of a total of 22 fetuses screened, five were identified as being heterozygous (+/–) for the polymorphism and thus informative. For fetuses F10 and F17, maternal DNA was homozygous for the undigested allele (–/–), whereas fetuses F7, F8 and F20, maternal DNA was heterozygous. RNA was extracted from all tissues available from these five

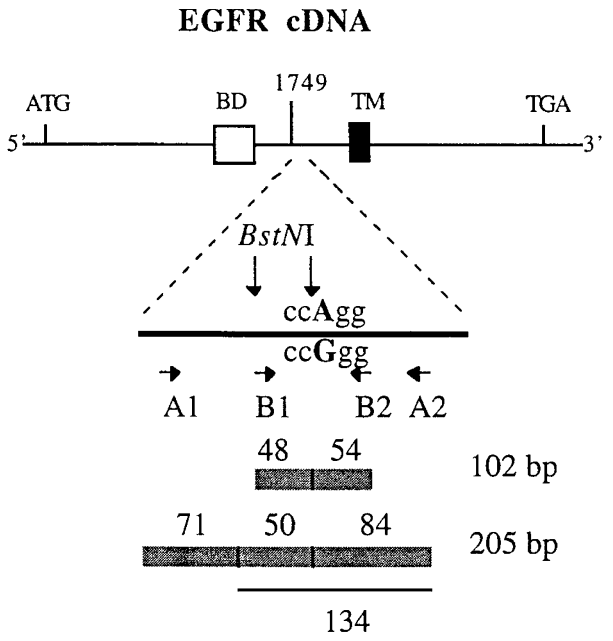

Figure 2 *Schematic map of* EGFR *cDNA (adapted from Moriai* et al[22]*) showing the transition site which creates a* BstN*I restriction site. ATG and TGA represent the start and stop codons. The open box represents the ligand binding domain (BD). The black box represents the transmembrane domain (TM). Locations of the PCR primers are indicated by horizontal arrows. The grey bars represent the amplified fragments with sizes of digested products indicated by the black divisions. Note that an individual homozygous for presence of the* BstN*I cutting site at position 1749 will show three bands of 50, 71, and 84 bp using primer pair A1/A2. An individual homozygous for the absence of the site will show two bands of 71 and 134 bp. A heterozygote with biallelic expression will have four bands of 50, 71, 84 and 134 bp*

fetuses and reverse transcribed. Primers A1 and A2, which span an intron, were used to amplify the region of interest and the products again digested with *BstN*1. The resultant products were digested with *BstN*I. The presence of both alleles was seen in all five cases in a range of different tissues (Figure 3 and Table 1). Expression of the *EGFR* gene is therefore biallelic in both first and early second trimester fetal tissues.

Expression of EGFR *in SRS Patients with mUPD7*

The expression of *EGFR* was also investigated in fibroblast and transformed lymphoblast cells from SRS patients. RNA was obtained from two normal individuals, two SRS patients (HW and GB) with mUPD7 and three SRS patients (JC, MA and TL) without mUPD7. RT-PCR was then used to study *EGFR* mRNA expression in these individuals using primers EGFR-F and -R. PCR products of the expected size (208 bp) were seen in both UPD and non-UPD patients, as well as in the normal controls (Figure 4). Similar results were obtained using transformed lymphoblast cells

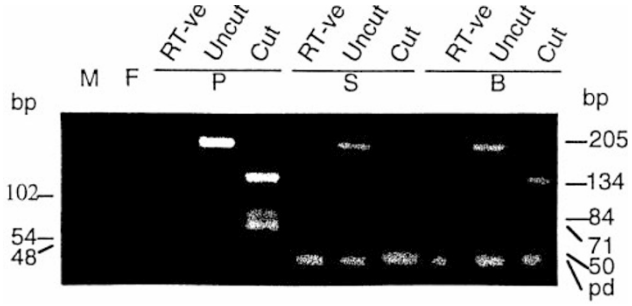

Figure 3 *Demonstration that* EGFR *is biallelically expressed in placenta (P), skin (S) and brain (B) from heterozygous fetus, F17. Genomic DNA from the homozygous mother (M) and heterozygous fetus (F) has been amplified with primer pair B1/B2; cDNA has been amplified with primer pair A1/A2. The use of primers A1/A2 which span an intron, provides a control for the absence of genomic contamination. This is additionally confirmed by the RT -ve control lanes. Restriction digest cuts the 205 bp fragments (A1/A2) to give four bands, as expected if both parental alleles are expressed. Primer-dimer band (pd) visible for primer pair A1/A2*

Table 1 Biallelic expression of the *EGFR* gene in five heterozygous fetuses

	F7	*F8*	*F10*	*F17*	*F20*
Placenta	Biallelic	Biallelic	Biallelic	Biallelic	NA
Skin	Biallelic	Biallelic	Biallelic	Biallelic	Biallelic
Brain	Biallelic	Biallelic	NA	Biallelic	Biallelic
Kidney	NA	NA	Biallelic	Biallelic	Biallelic
Heart	NA	NA	Biallelic	Biallelic	NA
Gut	Biallelic	Biallelic	Biallelic	Biallelic	Biallelic
Lung	NA	Biallelic	NA	NA	Biallelic
Liver	Biallelic	Biallelic	NA	NA	NA

Foetuses F7, F8, F10, and F17: all 9–10 week gestation; fetus F20: 14 week gestation. NA: not available for study.

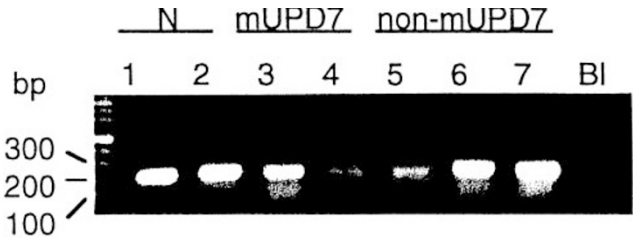

Figure 4 *Gel electrophoresis of RT-PCR products, showing* EGFR *mRNA expression in cultured fibroblasts. Lanes 1 and 2: normal individuals; lanes 3 and 4: SRS patients HW and GB with mUPD7; lanes 5, 6 and 7: SRS patients JC, MA and TL without mUPD7; Bl: blank (no template)*

(data not shown) although these cells did not express *EGFR* as highly.

Discussion

EGFR lies within a region on chromosome 7p12-13 homologous to an imprinted region in the mouse and its function is thought to be closely linked with fetal growth. It is therefore an obvious candidate for a role in the SRS phenotype associated with mUPD7. We have demonstrated that in a number of different, first and early second trimester, fetal tissues *EGFR* is biallelically expressed. The absence of evidence for imprinting of this gene makes its involvement in SRS unlikely.

Contamination of fetal samples by maternal tissue could potentially obscure an imprinting effect. However, paternal imprinting could not have been missed in those two fetuses (F10 and F17) whose mothers were homozygous for the undigested allele. Studies of maternal/fetal pairs where the mother was heterozygous and the fetus homozygous show evidence of amplification from contaminating maternal mRNA in two out of five tissues investigated. In both these cases the maternally derived bands were significantly fainter (data not shown). The consistent demonstration of biallelic expression in a large number of tissues from four different individuals indicates that contamination is not obscuring maternal imprinting.

Several studies have described tissue specific imprinting of certain genes.[19] Such findings make absolute exclusion of a gene as a candidate, on the basis of lack of observable imprinting, difficult. For example, it was initially suggested that *UBE3A*, recently identified as the Angelman syndrome gene,[26] was an unlikely candidate for the same disorder as expression had been demonstrated from both parental alleles in cultured fibroblasts and lymphoblasts. It is therefore important to study parental allele expression in those tissues likely to be involved in the pathogenesis of the disease being investigated. Adequate placental function is an important determinant of fetal growth. Crucially, we have shown that the *EGFR* gene is biallelically expressed in placental tissue.

The imprinting status of certain genes has also been shown to alter during the course of development.[20] Unfortunately, although fetuses up to 18 weeks' gestation were screened, the latest stage informative fetus was of 14 weeks' gestation. Thus definition of imprinting status was confined to first and early second trimester fetal tissues. However, demonstration of *EGFR* expression in fibroblasts and lymphoblasts from patients with mUPD7 also rules out maternal imprinting of *EGFR* in these cells in childhood. This strengthens the evidence that *EGFR* is biallelically expressed throughout development.

The study of gene expression in SRS patients with mUPD7 provides a potentially useful means of identifying maternally imprinted genes on chromosome 7. However, imprinting status may alter postnatally as well as during cell culture and transformation. One must therefore always remain cautious about excluding genes as potential candidates on the basis of such observations alone. For example, it has recently been demonstrated that human *PEG1/MEST* is expressed from the paternal allele alone in several different human fetal tissues.[27] This gene maps to chromosome 7q32 and has therefore also been proposed as a candidate for the SRS. However, Riesewijk *et al* also demonstrated *MEST* transcripts in a lymphoblastoid cell line with mUPD7, and biallelic expression in adult blood lymphocytes. In this investigation we have chosen to analyse *EGFR* expression in both transformed lymphoblastoid cell lines and cultured fibroblasts from SRS patients. Fibroblasts were studied to address concerns about artifactual loss of imprinting in transformed cell lines.

We are aware that there are other growth-related genes on chromosome 7 which also deserve investigation as candidate imprinted genes. Within the same region 7p12-13, homologous to an imprinted region on mouse proximal chromosome 11, lie *IGFBP1* and *IGFBP3*. These code for insulin-like growth factor binding proteins 1 and 3, integral members of the cell growth-regulating insulin-like growth factor axis.[28] We are planning a similar investigation, using polymorphisms within the coding region of these genes, to define their imprinting status.

Until now there have been only limited descriptions of *EGFR* expression in human fetal tissues[10–13] though its ligands have been shown to have multiple roles during development. Despite its name, numerous cell culture studies have shown that EGF is a potent mitogen for a wide variety of cell types of ectodermal, mesodermal and endodermal origin.[29] It promotes proliferation in developing tissues such as tooth extracts.[30] In addition, a number of other functions likely to be important in developmental processes have been demonstrated. These include stimulation of differentiation of several different cell types, including type II pneumocytes in lung epithelium;[31] increased cell

motility;[32] synthesis of extracellular matrix proteins[33] and stimulation of angiogenesis.[34] The biological activity of EGF is also shared by TGFα and in many responses TGFα has been found to be more potent.

Given the wide-ranging effects of both its ligands, the large number of tissues in which the EGFR has been reported to be expressed in the mouse[8] is unsurprising. We have shown that *EGFR* mRNA is also detectable in a wide range of tissues from different developmental origins during both first and second trimester in man. These results lend further weight to the view that EGFR and its ligands have important, and probably numerous, roles to play in normal human fetal growth and development. Closer investigation of these functions should help elucidate the mechanisms by which EGFR is related to normal and abnormal patterns of fetal growth.

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