Overexpression of the Axl tyrosine kinase receptor in cutaneous SCC-derived cell lines and tumours

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The molecular mechanisms that underlie the development of squamous cell skin cancers (SSC) are poorly understood. We have used oligonucleotide microarrays to compare the differences in cellular gene expression between a series of keratinocyte cell that mimic disease progression with the aim of identifying genes that may potentially contribute towards squamous cell carcinoma (SCC) progression in vivo, and in particular to identify markers that may serve as potential therapeutic targets for SCC treatment. Gene expression differences were corroborated by polymerase chain reaction and Western blotting. We identified Axl, a receptor tyrosine kinase with transforming potential that has also been shown to have a role in cell survival, adhesion and chemotaxis, was upregulated in vitro in SCC-derived cells compared to premalignant cells. Extending the investigation to tumour biopsies showed that the Axl protein was overexpressed in vivo in a series of SCCs.

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MATERIALS AND METHODS

Cell culture

Early passage (less than p16) PM1, MET1 and MET4 keratinocyte cell lines were grown with an irradiated fibroblast feeder layer in Dulbecco’s modified Eagle’s medium plus HAMS F12 medium containing 10% foetal calf serum essentially as described (Rheinwald and Green, 1975).

Microarray experiments and semi-quantitative reverse transcription–polymerase chain reaction

Briefly, total RNA was extracted from 70–80% confluent PM1, MET1 and MET4 keratinocyte cell lines using TRIzol reagent (Invitrogen-15596, Paisley, UK) and was used to generate labelled probes as per manufacturer’s instructions. These were used to hybridise the U133A Gene Chips using an Affymetrix Fluidics station 400. Three biological replicates were performed for each of the three cell lines. Full details of the methodology and statistical analysis can be found in Supplementary Material.
Table 1  Gene expression profile using Affymetrix arrays of genes differentially expressed in MET1 and MET4 vs PM1 cell line and MET1 vs MET4.

| Genbank     | Common     | Function                                      | MET1 vs PM1 | MET4 vs PM1 | MET1 vs MET4 |
|-------------|------------|----------------------------------------------|-------------|-------------|--------------|
| Genbank     | Common     | Common                                       | Fold +/-    | Fold +/-    | Fold +/-    |
| Ubiquitin cycle/proteolysis and peptidolysis | CTSB       | Cathepsin B                                  | -5          |             |              |
|             | AQP3       | Aquaporin 3                                  | -10         | -6          |              |
|             | N4507      | Nuclear transport factor 2-like export factor 2 | 8           |             |              |
| Transcription/replication | CEBPA      | CCAAT/enhancer binding protein (C/EBP), alpha | -10         |             |              |
|             | HOXAI      | Homeo box A1                                 | 6           | 8           |              |
|             | NSBP1      | Nucleosomal-binding protein                   | 16          |             |              |
|             | FKBP1      | FK506-binding protein 11, 19kDa              | 5           |             |              |
|             | AOX1       | Aldehyde oxidase 1                            | 8           |             |              |
| Signaling   | NRGN       | Neurogranin (protein kinase C substrate, RC3) | 11          |             |              |
|             | DTR        | Diphtheria toxin receptor                     | 16          | 22          |              |
|             | PDAPI      | Homo sapiens, clone IMAGE:3457786             | -8          |             |              |
|             | FYN        | FYN oncogene related to SRC, FGR, YES        | 5           |             |              |
|             | BST2       | Bore marrow stromal cell antigen 2            | 16          |             |              |
|             | EMR2       | epr-like, hormone receptor-like sequence 2    | -6          |             |              |
|             | SGNE1      | Secretory granule, neuroendocrine protein 1   | 5           |             |              |
|             | TXNIP      | Thioredoxin interacting protein               | -5          |             |              |
| Development | TPM1       | Tropomyosin 1 (alpha)                         | 6           |             |              |
|             | ZC1        | Zic family member 1 (odd-paired homolog, Drosophila) | 33          | 39          |              |
|             | JAG1       | Jagged 1 (Agiage syndrome)                    | 5           |             |              |
|             | EVC        | Ellis van Creveld syndrome                    | 10          |             |              |
| Growth factors/chemokine/cytokine/inflammation/immune response | PLAU       | Plasminogen activator, urokinase             | 6           |             |              |
|             | C1R        | Complement component 1, r subcomponent       | 13          | 10          |              |
|             | CTGF       | Connective tissue growth factor               | 13          |             |              |
|             | TPST1      | Tyrosylprotein sulfotransferase 1             | 8           |             |              |
|             | BLNK       | B-cell linker                                | 6           |             |              |
|             | SI0GA8     | S100 calcium-binding protein A8 (calgranulin A) | 5           |             |              |
|             | PTX3       | Pentaxin-related gene, rapidly induced by IL-1 beta | 8           |             |              |
|             | M9176      | CEACAM1                                      | 6           |             |              |
|             | SEMA3C     | Semaphorin 3C                                | 10          |             |              |
|             | GBPI       | Guanylate-binding protein 1, interferon-inducible, 67kDa | 7           |             |              |
|             | ANKRD1     | Cardiac ankrin repeat protein                 | -8          |             |              |
|             | PTG52      | Prostaglandin-endoperoxide synthase 2         | 5           | 16          |              |
|             | TPR2       | Tissue factor pathway inhibitor 2            | 9           |             |              |
|             | CXCL5      | Chemokine (C-X-C motif) ligand 5             | 227         |             |              |
| Cell cycle/oncogene/tumour suppressor | CCND2      | Cyclin D2                                    | -7          | 8           |              |
|             | IL1A       | Interleukin 1, alpha                          | 5           |             |              |
|             | BCAT1      | Branched chain amidotransferase 1, cystolic    | 11          |             |              |
|             | COL5A2     | Collagen, type V, alpha 2                     | 44          | 23          |              |
|             | IL8        | Interleukin 8                                 | 112         | 32          |              |
| Metabolism/stress response | MYLK       | Myosin, light polypeptide kinase              | -7          | 7           |              |
|             | OAS1       | U5'-oligoadenylate synthetase 1, 40/46kDa     | 5           |             |              |
|             | UROD       | Uroporphyrinogen decarboxylase                | 16          |             |              |
|             | SC4MOL     | Sterol-C4-methyl oxidase-like                 | 7           |             |              |
|             | FADS2      | Fatty acid desaturase 2                       | 4           | 5           |              |
|             | LPLREL1    | Leprecan-like 1                               | 10          | 15          |              |
|             | DNAJ      | DnaJ (Hsp40) homolog, subfamily B, member 4   | 5           |             |              |
|             | TM4SF7     | Transmembrane 4 superfamily member 7          | -5          | -6          |              |
|             | ASS        | Arginine:ornasucinate synthetase              | -5          |             |              |
| Cytoskeleton/cell adhesion | PPN2       | Profilin 2                                    | 25          | 15          |              |
|             | KRT8       | Keratin 8                                    | -6          |             |              |
|             | KRT6B      | Keratin 6B                                   | 5           |             |              |
|             | ITGB1      | Integrin, beta-like 1 (with EGF-like repeat domains) | 7           |             | 15           |

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Quantitative reverse transcription–polymerase chain reaction (RT—PCR) was performed to validate data from GeneChip experiments and was performed using an OPTICON ™ 2 Continuous Fluorescence Detection System (Bio-Rad) on the three replicate samples used in the GeneChip analysis. Full details of the Q–PCR conditions are given in Supplementary Material.

The primer pairs used for amplification of the selected targets were:

- **Axl**: GAGAACATTAGTGCTACGCGGAA/CCTTAGCCCTATGTCCATTAGCA
- **G6PDH**: GTTCCGTGAGGACCAGATCTAC/GGCTCCTTGAAGGTGAGGATAA

**Antibodies and immunohistochemistry**

Antibodies used in this study were anti-Axl (C-20), anti-Gas 6 (N-20), (Santa Cruz Biotechnology Inc., Palo Alto, CA, USA), anti-α tubulin (Ab-1, Oncogene Science, Cambridge, MA, USA).

Archival paraffin blocks were used for immunohistochemistry; ethical approval for this study was obtained from the East London and City Health Authority Research Ethics Committee.

Axl expression was examined using standard immunohistochemical techniques using 4 μm thick sections that had been deparaffinized, then blocked with rabbit serum and incubated with a goat polyclonal anti-Axl antibody. The sections were then processed as described (Jackson et al., 2002). Quantitative analysis of Axl staining was performed using KS400 version 3.0 imaging software (Carl Zeiss Ltd, Welwyn Garden City, UK). The percentage of cells expressing Axl was calculated in four representative high-power fields. Statistical analysis was performed using Arcus Quickstat (Statsdirect, Sale, UK, Biomedical version 1.1) and JMP (SAS Inc., Cary, NC, USA). For Western blot analysis was performed as described (Jackson et al., 2002), signals were detected using ECL + (Amersham Biosciences UK Ltd, Bucks, UK).

**RESULTS**

Gene expression profiles in premalignant and malignant keratinocyte lines

We compared the relative gene expression levels of the MET1 and MET4 lines with PM1, and also compared expression in MET4 with the solid tumour from which it appears to have originated, MET1 (Popp et al., 2000). The profiling revealed that 276 genes were statistically differentially expressed in PM1 cells compared to MET1 and MET4 cells ($P = 0.0001$). For practical reasons, we have applied an arbitrary filter level of five-fold changes in the ratio of expression levels; this relatively high cutoff point was used with a...
Axl mRNA and protein expression in PM1, MET1 and MET4 cell lines

Quantitative RT–PCR was performed on axl transcripts to support the findings of the expression profiling. The analysis was carried out on the RNA prepared for the three biological replicates used in the Affymetrix analysis. The results shown in Figure 1A support the data from the chip analysis. Western blotting of cell lysates separated by SDS–PAGE and Western blotted using specific monoclonal antibodies as described in Materials and Methods. Equal loading of proteins was verified by Western blotted of tubulin.

Figure 1  (A) Quantitative RT–PCR of axl gene expression in PM1, MET1 and MET4 cells. (B) Expression of Axl and Gas6, in PM1, MET1 and MET4 cells. Protein extracts were prepared from the different cell lines, separated by SDS–PAGE and Western blotted using specific monoclonal antibodies as described in Materials and Methods. Equal loading of proteins was verified by Western blotting of tubulin.

view to focus on the genes that are most grossly affected. As a result, an overall comparison of transcript levels from PM1 vs MET1, PM1 vs MET4 and MET1 vs MET4 revealed that 82 genes were significantly differentially expressed with a greater than five-fold change across the three tumour-derived cell lines that fell into diverse functional categories potentially affecting extracellular and intracellular signalling, proliferation and adhesion (Table 1). In particular, we noted that the axl tyrosine kinase receptor was significantly overexpressed in the MET1 relative to PM1 cells, and was also overexpressed 4.3-fold in MET4 relative to PM1 cells (Table 1).

Axl mRNA and protein expression in PM1, MET1 and MET4 cell lines

Immunohistochemical analysis of Axl expression in SCCs

To evaluate the expression of Axl in tumours, we performed an immunohistochemical study on a panel of SCCs, BCCs and normal skin biopsies using anti-Axl-specific antibodies. Axl expression was examined in 17 SCCs (11 well-differentiated and six poorly differentiated) from 16 individuals (Figure 2). Axl expression in 10 BCCs and nine normal skin samples was also investigated. Mast cells that showed consistent, strong, cytoplasmic staining were used in all sections as a positive internal control (data not shown). Goat IgG, at the same concentration as the anti-Axl goat IgG, served as a negative control. Normal epidermis had almost no staining (see Figure 2D) with a mean of 1.3% (95% confidence interval (CI): 0.3 – 2.3) of epidermal cells staining in each section examined. The mean percentage of cells staining with Axl in BCC was 1.3% (95% CI: 0.5 – 2.1%), suggesting that Axl does not have a significant role in cell signalling in BCC (see Figure 2E).

In contrast to normal skin and BCC, 13 out of 17 SCCs (76%) had significant Axl expression. The mean percentage of well-differentiated SCC (SCCW) cells staining with Axl was 21.5 (95% CI: 5.2 – 37.8%). In general, SCC tumour cells exhibited cytoplasmic staining, although there were a few SCC sections where membranous staining of individual cells was detectable (see Figure 2A). Furthermore, one section showed clear heterogeneity in staining within the SCCW (Figure 2B). The poorly differentiated SCC (SCCP) (Figure 2C) group displayed less Axl staining than SCCW, with a mean percentage of cells staining of 10.7% (95% CI: 1.2 – 22.6%). Statistical analysis was performed using Dunn’s Method to compare Axl staining in normal skin and tumours. There was a statistically significant difference between well- and poorly differentiated SCC compared to normal skin ($P<0.01$). There was no significant difference between BCC and normal skin staining with Axl, suggesting that Axl signaling may be restricted to SCCs.

DISCUSSION

The deregulation of cellular signalling networks underpins much of the basic framework of carcinogenic processes. A striking feature of the expression analysis was the finding that the axl gene was greatly upregulated in the MET1 cells compared to PM1. Overexpression of both the mRNA and protein was confirmed in MET1 cells in subsequent experiments. Our results are supported by previous studies in murine SCC where increased axl expression was also noted (Loercher et al, 2004). For this analysis, we have exclusively focused on genes whose expression was altered more than five-fold. We cannot rule out at this stage that genes whose expression was not changed by more than five-fold between the different cell lines used in this study may play an important role in determining not only the phenotype of these cells, but also in tumour progression in vivo.

We then extended the observations on the cell lines to investigate Axl protein expression in a pilot study of SCC biopsies. This analysis showed that Axl expression was increased in a significant proportion of the tumours analysed relative to normal skin. Although this is a small series of tumours, it not only validates the approach of using the PM and MET cell lines as a model system, but also suggests that Axl may be a novel marker whose overexpression is frequently associated with SCC. Whether Axl is also overexpressed in other precursor lesions such as actinic keratoses remains to be investigated. Axl expression was not observed in BCC biopsies, suggesting that Axl is not involved in altering signal transduction pathways in these tumours. Axl overexpression has also been noted previously in a variety of other cancers including ovarian (Sun et al, 2004), ocular melanoma (van Ginkel et al, 2004), osteosacroma (Nakano et al, 2003) and renal (Chung et al, 2003) tumours. Axl has been shown recently to play an important role in cell migration and proliferation of human endothelial cells (Holland et al, 2005). Receptors such as Axl that modulate a number of cellular processes such as growth, adhesion and migration and that are overexpressed on cancer cells, makes them targets for the development of novel therapeutics. Multiple clinical trials have employed novel strategies including antibodies that are antagonistic to such receptors (Finn and Slamon, 2003; Emens and Davidson, 2004; Mineo et al, 2004), or alternatively, low molecular weight inhibitors of the kinase activity have also been used, including imatinib mesylate (Gleevec) (Druker, 2004; Puslipher, 2004; Ross and Hughes, 2004) and gefitinib (Iressa) (Wakeling et al, 2002; Blackledge, 2003; Black-
Combination therapy, using both monoclonal antibody together with drug treatment, has also been evaluated (Huang et al., 2004). As yet, no specific inhibitors of Axl activity have been described. Our results suggest that further studies aimed at further elucidating the potential role of Axl in SCC are merited, as it may represent a potential therapeutic target for intervention in skin cancer development.

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