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Detection of Epidermal Growth Factor Receptor Gene Amplification in Human Squamous Cell Carcinomas Using Fluorescence *in situ* Hybridization

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The gene for epidermal growth factor receptor (EGFR) is associated with development of certain human cancers. In this study, we employed the improved fluorescence *in situ* hybridization technique to detect EGFR gene amplification in cell lines and tissue sections from human squamous cell carcinomas. We detected multiple distinct signals as arrayed amplicons on metaphase chromosomes and interphase nuclei of tumor cells. Our results provide a basis for rapid and quantitative DNA diagnosis of the EGFR gene amplification in individual cells of tumor specimens.

Key words: EGF receptor — Gene mapping — FISH — Gene amplification

EGFR[4] is a transmembrane glycoprotein with a molecular weight of 170,000 daltons and is localized on the surface of various types of mammalian cells.[1–4] EGFR is expressed generally in proliferating cells and its expression ceases when the cells achieve terminal differentiation.[5] Overexpression of the EGFR was initially found in an epithelial cell line (A431) of squamous cell carcinoma origin[6] and it was correlated with a unique marker chromosome by somatic cell hybridization studies.[7] EGFR has a significant similarity to a viral oncogene v-*erb*B producing tyrosine kinase activity, and thus is a subject of great interest in tumor molecular biology.[8]

The gene responsible for producing EGFR proteins was assigned to human chromosome 7, in the region of p13-qter, by the somatic cell hybridization technique[9,10] and later localized to the p14-p12 region of that chromosome using the radioisotope *in situ* hybridization technique.[11] A precise mapping of the EGFR gene to the middle of the band 7p12 using FISH was recently reported.[12]

Overexpression of EGFR was found at a significant rate in various tumors including squamous cell carcinoma of lung, esophagus, vulva and tongue[13–19] and amplification of the EGFR gene was found to be a major cause of the overproduction. Conventionally, the degree of EGFR gene amplification has been analyzed by Southern blot hybridization and found to be in the range of 2× to 40×.

In this study, EGFR gene amplification was examined by FISH in cell lines and tissue sections from squamous cell carcinomas.

Metaphase chromosome preparations were made from human diploid B-lymphoblastoid GM130B cells, epidermoid carcinoma A431 cells and NA cells as described previously.[20] The EGFR gene probe used was a mixture of cDNA plasmid clone pLsx and genomic DNA clone pERP8.[21] FISH was carried out as previously described with several modifications.[20] Briefly, the pLsx cDNA and genomic pERP8 DNA were individually labeled with digoxigenin-11-dUTP (BRL, Gaithersburg, MD) by nick translation. The reaction mixture (50 μl) contained 1 μg of plasmid clone DNA; dCTP, dATP, dGTP and digoxigenin-11-dUTP (20 m*M* each); DNA polymerase I (2 units, BRL); and DNase I (2 μg). Probe DNA (136.5 ng) was mixed with 10 μl of hybridization solution and used for one hybridization sample. Immunological detection of hybridization was carried out using FITC-conjugated sheep anti-digoxigenin antibody (Boehringer-Mannheim GmbH, Germany) and FITC-conjugated donkey anti-sheep immunoglobulin antibody (Silenus, Australia). Chromosome were counter-stained with PrI (10 ng/ml). The fluorescence image of probe hybridization was analyzed with a laser scanning microscope MRC-600 (Bio-Rad, Richmond, CA). FISH analysis of interphase cells and tumor sections was performed in the same way as described for metaphase chromosomes.

Metaphase chromosomes prepared from normal diploid lymphoblastoid cells were hybridized with a mixed DNA probe for the EGFR gene. The hybridization signal was amplified with FITC-conjugated antibodies

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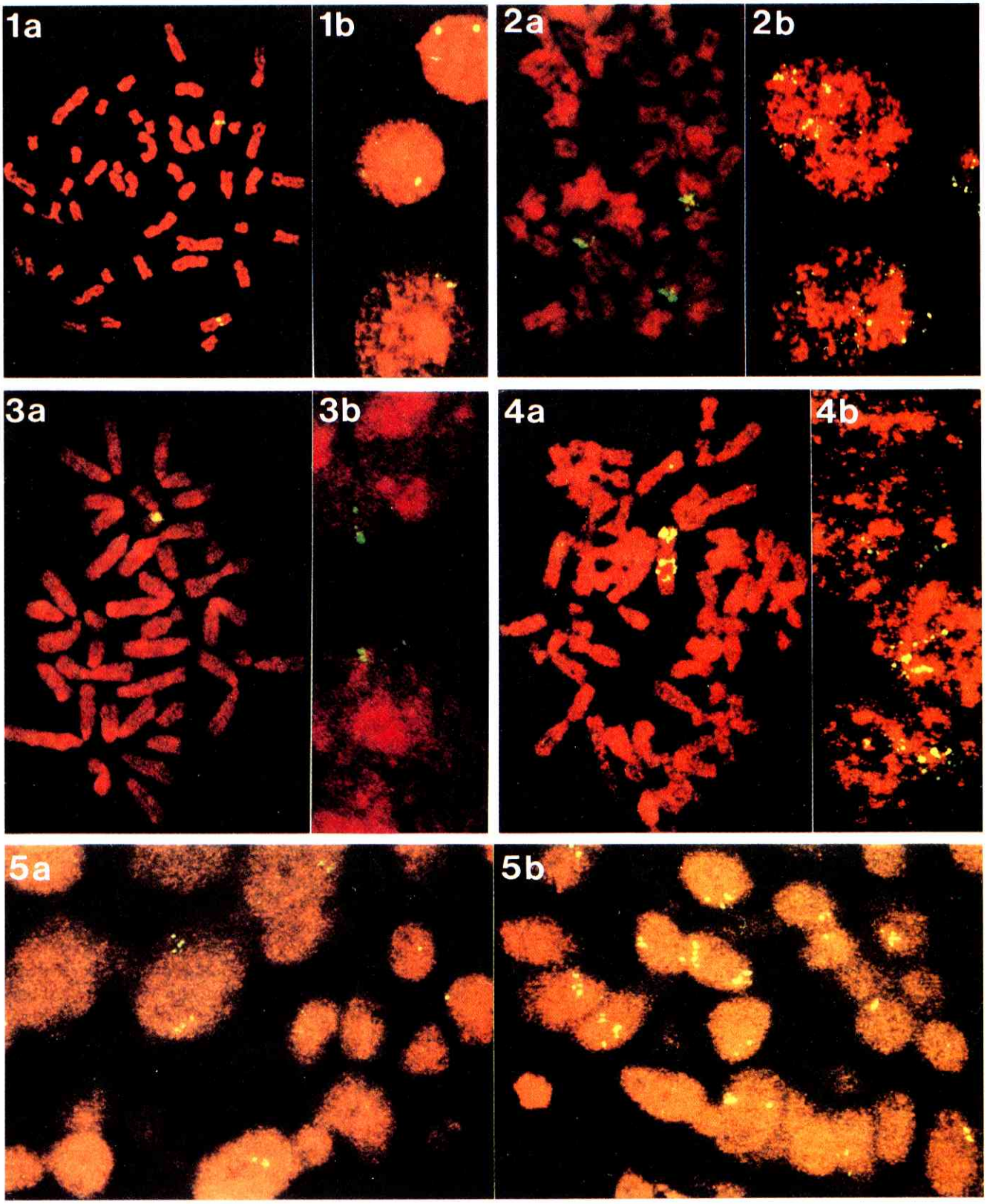
[4] The abbreviations used are: EGFR, epidermal growth factor receptor; FISH, fluorescence *in situ* hybridization; PrI, propidium iodide; FITC, fluorescein isothiocyanate.


1a
1b
2a
2b
3a
3b
4a
4b
5a
5b

and counter-stained with PrI. As seen in Fig. 1a, a pair of FITC signals was located on each of the short arms of two C group chromosomes. This same chromosome preparation was then Q-banded. Q-banding clearly showed that the signal-bearing chromosomes are chromosome 7 (data not shown). After detailed analysis of the data using computer software, the positions of the EGFR gene signals on the chromosomes were clearly located on the band p12 of chromosome 7.[12] When this improved FISH technique was applied to interphase cells, two to four distinct signals were detected, corresponding to the sub-stages of interphase of diploid cells (Fig. 1b).

It is well known that A431 cells contain abnormal chromosomes in which EGFR genes are substantially amplified.[11, 13, 14] In these A431 cells, there are three chromosomes, each of which exhibits a cluster of fluorescent signals (Fig. 2a). These signals are much more spread in the interphase nuclei as compared to metaphase chromosomes (Fig. 2b).

Previously, we produced a A431-mouse A9 hybrid cell line (AA1B2) which carries a marker chromosome M4 with amplified EGFR genes (Minoshima *et al.*, unpublished data). This AA1B2 hybrid clearly showed a single marker chromosome with multiple signals in metaphase among mouse chromosomes (Fig. 3a). These fluorescent signals seem aligned in the interphase nucleus (Fig. 3b).

NA is another tumor cell line with EGFR gene amplification.[22] In this cell line, EGFR gene amplification was detected on a single marker chromosome, where at least 15 fluorescent signals are countable (Fig. 4a and b). Thus, the degree of amplification of the EGFR gene can be clearly detected by FISH in metaphase chromosomes as well as interphase nuclei.

Finally, this FISH method was applied to a tissue section of neck squamous carcinoma, which was shown to overexpress EGFR by immuno-staining.[23] As can be seen in Fig. 5a and b, many cells in the tumor section exhibited multiple fluorescent signals, indicating the amplification of the EGFR gene.

Recently, we localized the EGFR gene to 7p12 by the FISH technique combined with an analysis using computer software.[12] In the present study, the FISH technique was used to detect the EGFR genes often amplified in tumor cells and tissues. In A431 cells, the EGFR hyperproduction was associated with EGFR gene amplification[14] and correlated with marker chromosome M4 involving chromosome 7.[11, 13] The marker chromosome M4 was responsible for the amplification of both intact and truncated EGFR genes.[14] In this study, three chromosomes including the marker chromosome M4 showed EGFR gene amplification.[11, 14, 15] The genomic size of the EGFR gene has been established to be about 110 kb[8] and the amplification of the EGFR gene was shown to take place together with large 5′- and 3′-flanking regions.[21] The size of amplified regions (amplicons) are as large as 1 Mb and the rearranged structures are unique in each tumor cell line.[21] Multiple distinct signals were observed in the interphase nuclei instead of large contiguous signals of amplified genes. This is reasonable because the FISH technique using the EGFR gene probe detects a single copy of this gene (~100 kb) which occupies only 1/10 of the amplicon (~1 Mb) and these amplicons are tandemly repeated, so signals are well separated along the amplified chromosomal region. In contrast to previous observations, A431 cells contained two abnormal chromosomes in addition to the marker chromosome M4 with multiple signals (amplified genes). This seems reasonable because the EGFR expression level in the mouse-human cell hybrid containing the marker chromosome M4 never reached the level of A431 tumor cells (Minoshima *et al.*, unpublished data). The additional chromosomes carrying amplified genes could also be responsible for EGFR hyperproduction. On the other hand, the amplified EGFR genes in NA cells were found on a unique aberrant chromosome which appears to be the MH1 de-

Fig. 1. Detection of EGFR gene localization by FISH. (a) FITC signals of EGFR gene on the PrI counter-stained metaphase chromosomes from normal diploid B-lymphoblastoid line GM130B. (b) FITC signals of EGFR gene on the interphase nuclei of GM130B cells.

Fig. 2. Detection of amplified EGFR gene by FISH. (a) Multiple FITC signals of EGFR gene on the PrI counter-stained metaphase chromosomes from epidermoid carcinoma A431 cell. (b) Multiple FITC signals of EGFR gene on the interphase nuclei of A431 cells.

Fig. 3. Detection of amplified EGFR gene by FISH. (a) Multiple FITC signals of EGFR gene on the PrI counter-stained metaphase chromosomes from AA1B2 hybrid cell. (b) Multiple FITC signals of EGFR gene on the interphase nuclei of AA1B2 hybrid cells.

Fig. 4. Detection of amplified EGFR gene by FISH. (a) Multiple FITC signals of EGFR gene on the PrI counter-stained metaphase chromosomes from epidermoid carcinoma NA cell. (b) Multiple FITC signals of EGFR gene on the interphase nuclei of NA cells.

Fig. 5. (a and b) Detection of EGFR gene amplification by FISH in a section of neck squamous cell carcinoma.

scribed in our previous report.[22] Based on these detailed studies using well-established tumor cell lines, we were able to detect the EGFR gene amplification in sections of tumor specimens. The results show that the FISH detection of amplified genes in the interphase nuclei will be useful for detection of oncogene amplification in individual cells of tumor specimens. This would provide a more quantitative DNA diagnosis than conventional techniques including Southern or spot blot hybridization.

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