Validation of gefitinib effectiveness in a broad panel of head and neck squamous carcinoma cells

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Abstract. Recently improved understanding of the pathogenesis of human head and neck squamous cell carcinoma (HNSCC) has led to the development of new, molecular-based therapeutic strategies, one of the more promising is the utilisation of tyrosine kinase (TK) inhibitors, targeting epidermal growth factor receptor (EGFR). In this study, we tested for gefitinib effectiveness in a broad panel of 12 newly established HNSCC cell lines, investigating its ability to reduce cell growth, to induce apoptosis and to modulate cell cycle and various EGFR pathway-related targets. Gefitinib IC_{50} values ranged between 0.064 and 33 μM, its capability to induce apoptosis and cell accumulation in G0/G1 phase was cell line-specific, and the main EGFR-related pathway involved in gefitinib activity was PI3K/Akt/mTor. We characterised our *in vitro* panel extensively, with the aim to identify predictive factors for gefitinib effectiveness; all cell lines were free of human papillomavirus infection, two were positive for Fhit expression, four expressed wild-type p53, and all of them variously expressed the other two p53 family members, p63 and p73. The comparison between the targets analysed and gefitinib effectiveness evidenced the absence of a clear relationship, excluding them as predictive factors for gefitinib efficacy. Our results confirmed the *in vitro* efficacy of an anti-EGFR approach, but other targets than those analysed here should be characterised in order to identify valid predictive factors for gefitinib utilisation.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the most frequently occurring cancers worldwide, and the overall survival period is very short as the cancer often metastasises in the lymph nodes of the throat (1,2). The failure of conventional chemotherapy, which does not increase the overall survival rate of patients, and the goal of identifying new biological targets for anticancer therapy, has stimulated the study of the molecular mechanisms of HNSCC progression (3-5). Among the promising new cellular targets, epidermal growth factor receptor (EGFR) seems to have a prominent role since it has been recognised as a prognostic factor in head and neck cancer (6,7). It was recently suggested that gefitinib (ZD1839; Iressa), an orally active, selective EGFR tyrosine kinase (TK) inhibitor (8) could be utilised in head and neck cancer therapy (9,10 and www.clinicaltrials.gov). This drug is a quinazoline derivative that selectively inhibits EGFR tyrosine kinase activity by binding the receptor at the ATP-site, and is in clinical use in lung cancer patients (11). The utilisation of gefitinib in preclinical studies and early clinical trials has recently shown evidence of its promising activity in head and neck cancer treatment (9,10,12). However, an important aspect that remains unclear in the utilisation of TK inhibitors relates to the difficulty of identifying any predictive factor that is applicable in all tumour types. It would therefore be of value to confirm the hypothesis that the efficacy of EGFR-targeting drugs in head and neck cancer is linked to EGFR tumour level, as recently suggested by Janmaat *et al* (13). Other biomarkers that have been suggested as predictive factors for gefitinib efficacy in other cancers, are increased EGFR gene copy number beyond EGFR protein expression, the presence of specific EGFR mutations and constitutive Akt activation (14-19).

In relation to other tumour types, an extensive pre-clinical analysis of the basic molecular mechanism involved in gefitinib-mediated anticancer effects in head and neck cancer is not available, e.g. in Shintani *et al* (20) gefitinib efficacy was investigated in only two HNSCC cell lines.

In this study, for the first time, the validation of gefitinib efficacy against head and neck cancer was performed in a broad panel of 12 newly established HNSCC cell lines and an extensive biological characterisation comprising cancer progression (including p53, Fhit, cell cycle characteristics and HPV infection) and EGFR-related markers (TK receptors, EGFR signal transduction pathway and ABCG2 expression), was also performed.

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Inactivating mutations of the p53 tumour suppressor gene are the most common genetic event identified for almost all human cancers, including HNSCC, and changes in p53 status have been linked to disease progression, decreased sensitivity to chemotherapeutic agents and poor prognosis (21). The key role of p53 in modulating EGFR expression (22-24) and in controlling cell proliferation and apoptosis poses the question of whether p53 status influences the efficacy of EGFR inhibitors. Moreover, the identification of two p53 homologues, p63 and p73 (25) with high identity of their amino acid sequence (about 63% in the DNA-binding domain) suggested a redundant function in the regulation of gene expression for these three transcription factors. p73 can activate p53-regulated genes, suppress growth or induce apoptosis (26). Furthermore, TAp63 and ΔNp63, two p63 isoforms, perform opposite functions, inducing cell-cycle arrest and cell proliferation respectively; the latter is frequently expressed in HNSCC (27).

Another factor analysed was Fhit (fragile histidine triad) which is frequently altered in head and neck cancer progression (28). It was recently demonstrated that the activation of EGFR family members induced Fhit phosphorylation by Src and the subsequent proteasome degradation of the phosphorylated Fhit protein (29), suggesting a key role for Fhit in the balance of proliferation/survival/apoptosis signals and as a hypothetical predictive biomarker for gefitinib efficacy.

Another biological factor that has to be considered in head and neck cancer treatment with gefitinib, is human papillomavirus (HPV) status; HPV infection has been closely linked to oral cancer, with 30-40% of oral cancer biopsy specimens containing viral DNA of the high-risk HPV types, such as HPV-16, -18 and -33 (30,31).

The *in vitro* model was also characterised for the molecular alterations and expression of TK receptors and their downstream effectors (32). The main EGFR down-stream effectors analysed were AKT and Erk1/2, involved in the survival and proliferation pathways respectively, and the tumour suppressor gene, PTEN, as its inactivation can induce resistance to TK inhibitors (33-35). The drug efflux pump ABCG2 (BCRP/MXR/ABCP) was also analysed in relation to gefitinib effectiveness in other cancer models (36-39). Finally, all HNSCC cell lines were analysed for the presence of mutations in the region of the EGFR gene coding for the tyrosine kinase domain (exon 18 to exon 21) and in the ATPase domain (exon 27 and 28) (18).

Materials and methods

Reagents. Gefitinib was provided by AstraZeneca Pharmaceuticals (London, UK). Stock solutions were prepared at 20 mM in dimethyl sulfoxide (DMSO) and stored in aliquots at -20˚C. The monoclonal antibody anti-EGFR was provided by BD Transduction Laboratories (USA); anti-ß-actin antibody was purchased from Sigma (Milan, Italy); anti-phosphotyrosine PY99, anti-Fhit, anti-PTEN, anti-VEGFR2, and anti-ß-tubulin antibodies were provided by Santa Cruz Biotechnology (USA); anti-AKT, anti-phospho-AKT, anti-ERK1/2 and anti-phospho-ERK1/2 were provided by Cell Signaling (USA); anti-ABCG2 (human) MAb (BXP-21) by

Alexis Corporation; and p53 polyclonal antibody NCL-CM1 and p63 polyclonal antibody were from Novocastra. A mouse and a rabbit HRP (Amersham Pharmacia Biotech, Uppsala Sweden) were used as secondary antibodies.

Establishment of human HNSCC cell lines. The excised tumour masses from different tissues of the head and neck region consisted of larynx (one case, HNC-150), oral cavity (six cases, HNC-91, -97, -124, -160, -199, -212), tonsil (three cases, HNC-41, -206, -211), hypopharynx (one case, HNC-180) and paranasal sinus (one case, HNC-136). These were minced and plated on dishes for explantation culture in DMEM supplemented with 20% FBS, 50 ng/ml of EGF, 10 μg/ml of hydrocortisone, and 5 μg/ml of spermine. The tissue samples were obtained from cancer patients during oral surgery. Confluent primary cells were trypsinized and further cultured in DMEM supplemented with 10% FCS, 2 mM glutamine and 2 mM penicillin-streptomycin (Life Technologies), in a humidified incubator at 37˚C with an atmosphere containing 5% CO_2.

Evaluation of cytotoxicity. Determination of the IC_{50} was performed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assay. On day 1, 12,000 cells/well in a volume of 200 μl were plated in 96-well plates. In each plate, one column contained cells not exposed to drugs (control), and 5 columns contained cells exposed to increasing concentrations of gefitinib (from 10 nM to 100 μM) for three days. Each drug concentration was repeated in at least 6 identical wells. Gefitinib was added on day 2, and results were expressed as dose-effect curves with a plot of the fraction of unaffected (surviving) cells versus drug concentration. The IC_{50} was defined as the drug concentration yielding a fraction of affected (non-surviving) cells (0.5) compared with untreated controls. Each experiment was conducted in triplicate.

Cell cycle analysis. Cells were exposed to gefitinib, harvested, washed twice in ice-cold PBS (pH 7.4), fixed in 4.5 ml of 70% ethanol and stored at -20˚C for at least 24 h. After being washed once in ice-cold PBS, the pellet was re-suspended in PBS containing 1 mg/ml RNase and 0.01% NP40, and the cellular DNA was stained with 50 μg/ml propidium iodide (Sigma). Cells were stored in ice for 60 min prior to analysis. Cell cycle determinations were performed using a FACScan flow cytometer (Becton Dickinson), and data were interpreted using the CellQuest software, provided by the manufacturer. The DNA-ploidia pattern was evaluated comparing the shift of the G0/G1 peak of each cell line with respect to that of peripheral lymphocytes.

Determination of apoptosis. Exponentially growing cells were treated with gefitinib IC_{50} concentrations for 1-3 days. Apoptosis was evaluated using the Annexin V-FITC detection kit (BD Transduction) and by propidium iodide (PI) following the manufacturer's protocol.

Determination of human papillomavirus (HPV) infection. The presence of HPV (types 4, 5, 8, 9, 12, 14, 15, 17, 19-24, 26, 36-38, 47-50, 60 and 65) DNA was determined by

reverse line blot hybridisation previously described by Kleter *et al*, 1999; Melchers *et al*, 1999 and Quint *et al*, 2001. Oligonucleotide probes specific for 24 different HPV types were covalently attached to a membrane in parallel lines using a miniblotter. The templates used for analysis were DNA extracts previously labeled with biotin, using PCR. The labeled PCR products were then loaded onto the membrane in slots perpendicular to the oligo lines in order to allow all samples to interact with the HPV probes. Hybridisation took place in the miniblotter and was visualised using a peroxidase-labeled streptavidine, which interacted with the biotin of the PCR products, followed by chemiluminescence detection.

Western blot analysis. Total proteins were extracted from the cell culture by homogenisation in a radioimmunoprecipitation assay (RIPA) buffer [0.5 M NaCl, 1% Triton X-100, 0.5% NP40, 1% deoxycolic acid, 3.5 mM sodium dodecyl sulfate (SDS)], with a 20% protease inhibitor cocktail (Sigma, MO), and measured by the Bradford method. Proteins were electrophoretically separated by Western blotting and signals were detected by chemiluminescence assay. Expression levels were evaluated by densitometric analysis using Quantity One software (Bio-Rad, Hercules, CA) and the ß-actin expression level was used to normalise the sample values.

Immunoprecipitation assay. Cells were lysed in RIPA-IP buffer (140 mM NaCl, 20 mM Tris/HCl, 10 mM EDTA pH 8.0, 10% glycerol, 1% NP40, 1 mM Na-deoxycolic acid, 1 mM PMSF), passed through a 22-gauge syringe and cleared by centrifugation at 10,000 x g at 4˚C for 10 min. Proteins were immunoprecipitated by incubating 0.1-0.3 mg of total cell lysate with 0.2 µg of anti-phosphotyrosine antibody or EGFR antibody for 1 h at 4˚C. A/G agarose protein (2-5 µl) (Santa Cruz Biotechnology) was incubated overnight at 4˚C. The cell suspension was centrifuged at 2,600 rpm and the pellet was washed three times with PBS and then re-suspended in 10 µl of Laemmli buffer. Each sample was separated onto 10% acrylamide gel and Western blot analysis was performed as described above.

Reverse-transcription PCR. Each cell line was collected, the RNA was extracted with the Absolutely RNA Miniprep Kit (Stratagene) and 5 µg of RNA was converted to cDNA using the First Strand cDNA Synthesis Kit (Fermentas MBI). The primers used for the PCR were as follows: ΔNp73, 5'-ACC ATG CTG TAC GTC GGT GAC CCC-3' (forward) and 5'-GCG ACA TGG TGT CGA AGG TGG AGC-3' (reverse); ΔNp63, 5'-TGC CCA GAC TCA ATT TAG TGA G-3' (forward) and 5'-AGA GAG AGC ATC GAA GGT GGA G-3' (reverse); TAp63, 5'-GAC CTG AGT GAC CCC ATG TG-3' (forward) and 5'-CGG GTG ATG GAG AGA GAG CA-3' (reverse). As a control, the following human GAPDH was used: 5'-AAG GTG GTG AAG CAG GCG T-3' and 5'-GAG GAG TGG GTG TCG CTG TT-3'. Annealing temperatures and the number of cycles were specific for each isoform: ΔNp63, 25 cycles at 64˚C; TAp63, 35 cycles at 58˚C; ΔNp73, 40 cycles at 62˚C; and TAp73, 35 cycles at 61˚C. The rest of the PCR amplification consisted of 94˚C for 30 sec, for 30 sec, and 72˚C for 30 sec followed by

Table I. Primer sequences used for EGFR coding exon amplification.

Exon	Primer sequence
18	Fw 5' CAAATGAGCTGGCAAGTGCCGTGTC 3' Rv 5' GAGTTTCCCAAACACTCAGTGAAAC 3'
19	Fw 5' GCAATATCAGCCTTAGGTGCGGCT 3' Rv 5' CATAGAAAGTGAACATTTAGGATGTG 3'
20	Fw 5' CCATGAGTACGTATTTTGAAACTC 3' Rv 5' CATATCCCCATGGCAAACTCTTGC 3'
21	Fw 5' CTAACGTTCGCCAGCCATAAGTCC 3' Rv 5' GCTGCGAGCTCACCCAGAATGTCTGG 3'
27	Fw 5' CAACCTACTAATCAGAACCAGCATC 3' Rv 5' CCTTCACTGTGTCTGCAAATCTGC 3'
28a	Fw 5' CCTGTCATAAGTCTCCTTGTTGAG 3' Rv 5' GGTAGTCAGGGTTGTCCAGG 3'
28b	Fw 5' CGAGTATCTCAACACTGTCCAGC 3' Rv 5' CTAATGCGGGCATGGTG 3'

incubation at 72˚C for 7 min. The bands were visualised by ethidium bromide staining.

DNA sequencing analysis. Randomly primed cDNAs were reverse-transcribed from 5 µg of total RNA using First Strand cDNA Synthesis Kit in a 40-µl mixture. Two microlitres of the cDNA mixture was used to amplify the p53 transcript (exons 4-9). PCR products were cloned into PCR II using a TA™ Cloning Kit (Invitrogen, San Diego, CA) under the conditions recommended by the manufacturer. The nucleotide sequence of the cloned DNA was determined by a Taq Dideoxy Terminator Cyclic Sequencing Kit on an ABI 377 Automatic DNA Sequencer (PerkinElmer, Foster City, CA).

Mutational analysis of EGFR. Genomic DNA was extracted from each cell line, using QIAamp DNA Blood Midi Kit (Qiagen) following the manufacturer's instructions. DNA was quantified and mutational analysis was performed from EGFR exons 18 to 21 and in exons 27 and 28. EGFR coding exon was amplified by PCR using the primers described in Table I.

PCR amplification was carried out in a total volume of 50 µl containing 150 ng of genomic DNA as template, 1X reaction buffer, 1.5-3 mM magnesium chloride, 200 mM dNTP, 20-50 pmol of each PCR primer and 0.25 U AmpliTaq Gold. After PCR amplifications, 5 µl of the reaction product was analysed via gel electrophoresis and ethidium bromide staining. DNA sequencing was performed on both strands of two independent PCR products by cycle sequencing on an ABI PRISM 310 automated cycle sequencer, using the Terminator Cycle Sequencing Kit v.1.1 according to the manufacturer's protocol (Applied Biosystems,

Table II. Gefitinib effectiveness in HNSCC cell lines.

Cell line	Site of origin	3-day gefitinib treatment					
		IC_{50} (μM) $\pm$ SD	G0/G1 increase %	Apoptosis	p-EGFR expression %	p-AKT expression %	p-Erk1/2 expression %
HNC-211	Tonsil	0.064±0.01	9	+	ND	20	40
HNC-180	Hypopharynx	0.71±0.12	17	-	ND	10	0
HNC-97	Oral cavity	1.9±0.9	1	-	ND	0	50
HNC-97	Oral cavity	1.9±0.9	1	-	ND	0	80
HNC-124	Oral cavity	1.9±1.2	7	+	ND	0	ND
HNC-160	Oral cavity	2.2±1.1	1	-	ND	10	0
HNC-212	Oral cavity	4.22±1.8	9	-	80	0	10
HNC-212	Oral cavity	4.22±1.8	9	-	ND	0	10
HNC-91	Oral cavity	5.3±2.0	4	-	ND	100	100
HNC-41	Tonsil	5.4±1.6	0	-	ND	0	ND
HNC-206	Tonsil	8.3±2.4	22	-	80	30	100
HNC-136	Paranasal sinus	9.3±1.9	5	+	ND	ND	ND
HNC-199	Oral cavity	25±3.3	7	+	ND	100	100
HNC-150	Larynx	33±2.7	0	+	ND	30	100

CA). Whenever an alteration was identified, a new DNA aliquot from the same cell line was sequenced to confirm the result. The variants found in the sequence were characterised and compared to those in the online databases, EntrezSNP (http://www.ncbi.nlm.nih.gov) and Ensembl (www.ensembl.org).

Results

The *in vitro* validation of gefitinib effectiveness in head and neck cancer was performed in a broad panel of head and neck cancer cell lines. Firstly, we investigated the efficacy of gefitinib. We then analysed our *in vitro* model for several characteristics connected with cancer progression (including p53, Fhit, cell cycle characteristics and HPV infection), and the anti-tumour activity of EGFR inhibitors (TK receptors, EGFR signal transduction pathway, ABCG2 expression and the presence of mutations in the region of the EGFR gene coding for the tyrosine kinase and ATPase domains), with the aim of identifying predictive factors for this TK inhibitor activity.

Gefitinib effectiveness. A broad panel of 12 HNSCC cell lines was established from different tissues of the head and neck area and stabilised (Table II). This panel could be considered representative of head and neck cancer in *in vitro* studies.

The capability of gefitinib to inhibit cell growth was determined by incubating each HNSCC cell line with various drug concentrations for 3 days, and the results are reported as IC_{50} values in Table II. Out of all cell lines, HNC-211 cells were the most sensitive to the drug, with IC_{50} of 64 nM, and the most resistant ones were HNC-150 and -199 (IC_{50} of 25 and 33 μM, respectively). The other cell lines showed 10- to 300-fold greater resistance to the drug than HNC-211.
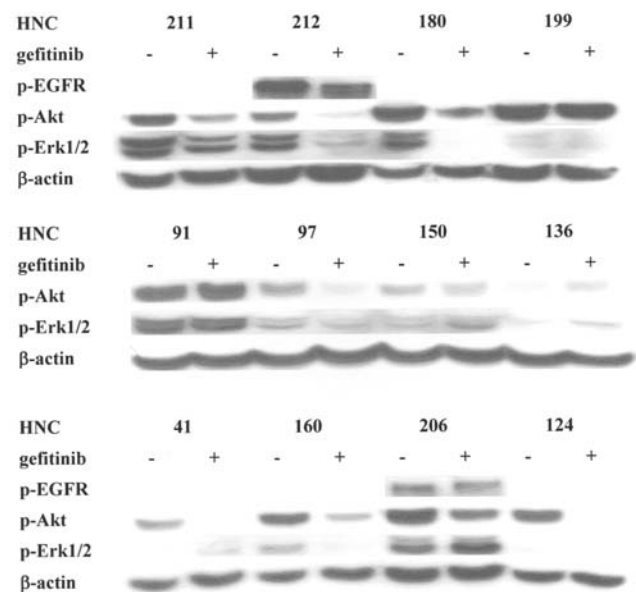

Figure 1. EGFR pathway modulation by gefitinib. HNSCC cells were incubated with gefitinib at IC_{50} concentration and the protein extracts were analysed by immunoprecipitation and/or Western blotting. In Western blot analysis, the amount of the different targets was determined using mono-clonal- or polyclonal- specific antibodies and ß-actin was used to normalise the values. p-EGFR was clearly detectable only in HCN-212 and HCN-206.

Then, the capability of gefitinib to induce apoptosis was investigated by treating all cell lines with gefitinib at IC_{50} concentration for 1-3 days. A 10-20% increase in Annexin V-positive cells was evident in only five cell lines: HNC-211, -124, -136, -199 and -150; the only difference among them was that the first two underwent apoptosis early (1-2 days) and this phenomenon decreased during the third day, while the

Table III. Characterisation of HNSCC cell lines.

Cell line	IC$_{50}$ (μM) ± SD	p53				ΔNp73	TAp73	ΔNp63	TAp63	p63	Fhit
		Exon	Codon base	Amino acid change	Protein expression					Protein expression	
HNC-211	0.064±0.01	6	177-183 Del 5	In frame	+	+	-	+	+	+	-
HNC-180	0.71±0.12	6	220 TAT-TGT	Tyr-Cys	-	-	-	+	+	+	-
HNC-97	1.9±0.9	5	180 GAG-AAG	Glu-Lys	-	+	-	+	+	+	-
HNC-97	1.9±0.9	7	248 CGG-TGG	Arg-Trp	-	+	-	+	+	+	-
HNC-124	1.9±1.2	(4-9)	WT	-	++	+	-	+	+	+	-
HNC-160	2.2±1.1	6	213 CGA-CGG	Arg-Arg	-	+	-	+	+	+	-
HNC-212	4.22±1.8	6	196 CGA-CCA	Arg-Pro	++	-	-	+	+	+	-
HNC-212	4.22±1.8	9	331 CAG-.AG	Frameshift	++	-	-	+	+	-	-
HNC-91	5.3±2.0	(4-9)	WT	-	-	+	-	-	+	-	+
HNC-41	5.4±1.6	(4-9)	WT	-	-	-	+	+	+	+	-
HNC-206	8.3±2.4	5	134 TTT-TCT	Phe-Ser	-	-	-	+	+	+	+
HNC-136	9.3±1.9	(4-9)	WT	-	+++	+	+	-	+	-	-
HNC-199	25±3.3	5	135 TGC-TTC	Cys-Phe	-	+	-	+	+	+	-
HNC-150	33±2.7	5	135 TGC-TAC	Cys-Tyr	-	+	-	+	+	+	-

last three showed progressive increase in Annexin V-positive cells as a function of time exposure (data not shown). The high variability of IC$_{50}$ values suggested the need to investigate the gefitinib activity in each cell line. In particular, the ability of gefitinib to modulate cell cycle progression was determined. A three-day drug exposure induced a slight blockage of the cell cycle at G0/G1 phase, with a maximum effect in HNC-206 inducing an increase in the accumulation of G0/G1 phase cells of ~22% (Table II).

The drug-dependent modulation of EGFR and its down-stream effectors, Akt and Erk1/2, was also investigated. Each cell line was treated with gefitinib at each IC$_{50}$ concentration for three days and the modulation of total and phosphorylated forms of EGFR, Erk1/2 and Akt was analysed. Gefitinib did not modulate the expression of EGFR, Akt and Erk1/2 (data not shown). Only HNC-212 and -206 cells showed detectable levels of p-EGFR expression and a 3-day gefitinib treatment showed 80% of residual EGFR in the phosphorylated form (Fig. 1). p-Akt was completely abolished by gefitinib in four cell lines, HNC-41,-124, -97 and -212, and variously reduced in the remainder, conversely p-Erk1/2 was reduced to a lesser extent with the exception of HCN-180 and -160, as reported in Fig. 1 and summarised in Table II.

Cell line characterisation. With the aim of identifying cellular or molecular factors that are predictive of gefitinib efficacy in our panel of head and neck cell lines, each cell line was characterised for targets related to cancer progression and drug activity.

In detail, expression and cell cycle distribution of Fhit and p53 family, were determined. As summarised in Table III, Fhit, the tumour suppressor that is frequently altered in head and neck cancer, was expressed only in two cell lines and a sequence analysis of exons 4-9 of the p53 gene provided evidence that 8 cell lines had mutated p53 and only four

cell lines, either wild-type and mutated (HNC-124, -136, -211, -212), exhibited detectable levels of p53. Other p53 homologues analysed in our *in vitro* panel were p63 (TAp63, ΔNp63) and p73 (TAp73, ΔNp73). A preliminary RT-PCR analysis of ΔNp73 showed that it was detectable only in HNC-150 and -211, while a following nested PCR evidenced its presence in 75% of all the cell lines (Fig. 2A). TAp73 was detectable only in HNC-41 and HNC-136 by both RT-PCR and nested PCR (Fig. 2B). Concerning p63 isoforms, ΔNp63 seemed to be overexpressed in eight cell lines by RT-PCR analysis, while nested PCR showed two more cell lines that were positive for this isoform expression (Fig. 2C). The high expression level of ΔNp63 in HNSCC cell lines was confirmed by Western blot analysis and the results are shown in Fig. 2E. The expression levels of TAp63 were also analysed by RT-PCR and nested PCR, and it seemed to be present in all cell lines (Fig. 2D). Cell cycle analysis showed that all cell lines had an atypical distribution in the G0/G1, S and G2/M phases and, with respect to lymphocytes, they were all aneuploid with a DNA index ranging between 1.13 and 1.75 (data not shown). Moreover, HNSCC cell lines analysed for the presence of HPV DNA (types 4, 5, 8, 9, 12, 14, 15, 17, 19-24, 26, 36-38, 47-50, 60 and 65) showed that it was absent (data not shown).

Our *in vitro* model was then analysed for several characteristics related to the mechanism of action of TK inhibitors, such as the expression levels of EGFR, p-EGFR, ErbB2, VEGFR-2, Erk1/2, p-Erk1/2, PTEN, Akt, and p-Akt. Higher levels of EGFR were observed in HNC-211, -199, and -206 cells whereas it was undetectable in HNC-91 and -136. Conversely, only HNC-206 and -212 showed detectable levels of p-EGFR (Fig. 3). The other two TK receptors, ErbB2 and VEGFR-2, and the two signal transduction pathway effectors, Akt and Erk1/2, were ubiquitously expressed in all cell lines (Fig. 3). Conversely, p-Akt and p-Erk1/2
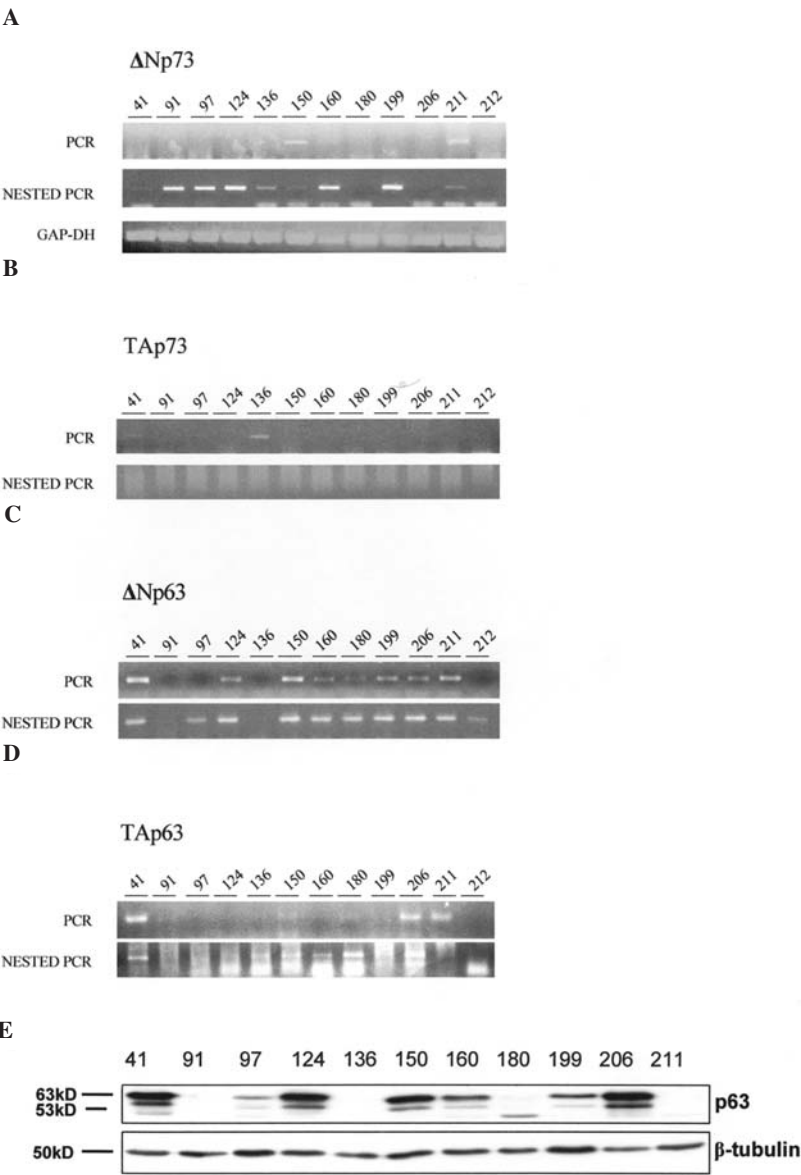
A

ΔNp73

41 91 97 124 136 150 160 180 199 206 211 212

PCR

NESTED PCR

GAP-DH

B

TAp73

41 91 97 124 136 150 160 180 199 206 211 212

PCR

NESTED PCR

C

ΔNp63

41 91 97 124 136 150 160 180 199 206 211 212

PCR

NESTED PCR

D

TAp63

41 91 97 124 136 150 160 180 199 206 211 212

PCR

NESTED PCR

E

41 91 97 124 136 150 160 180 199 206 211

63kD —
53kD —   p63

50kD —   β-tubulin

Figure 2. p63 and p73 characterisation in HNSCC cell lines. The isoforms of p73 [(A) ΔNp73, (B) TAp73] and p63 [(C) ΔNp63, (D) TAp63] and were analysed by PCR and nested PCR in all cell lines; protein was determined only for p63.

HNC   91   97   150   136   41   160   206   124   211   199   180   212

p-EGFR

EGFR

ErbB2
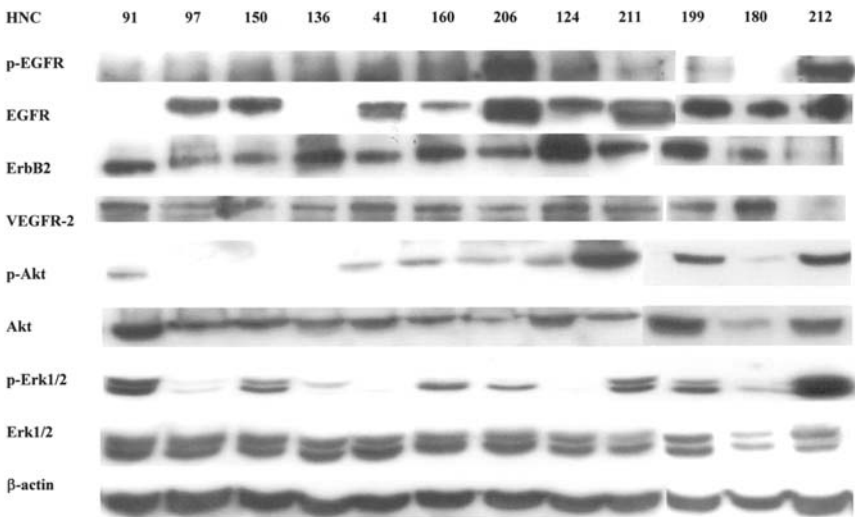
VEGFR-2

p-Akt

Akt

p-Erk1/2

Erk1/2

β-actin

Figure 3. TK receptor and downstream effector expression in HNSCC cell lines. EGFR, ErbB2, VEGFR-2, Erk1/2, p-Erk1/2, Akt and p-Akt expression in HNSCC cellular extracts was analysed by Western blotting. Phosphorylated EGFR was determined by immunoprecipitation followed by Western blotting.

Table IV. Characterisation of HNSCC cell lines.

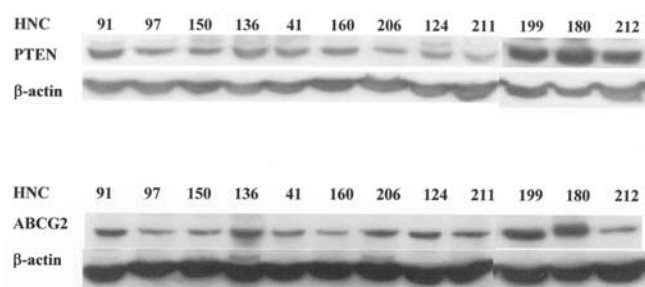| Cell lines	IC$_{50}$ (μM) ± SD	Basal protein expression %					
		EGFR	p-EGFR	p-Akt	p-Erk1/2	PTEN	ABCG2
HNC-211	0.064±0.01	+	+++	+++	++	+	+
HNC-180	0.71±0.12	-	++	-	+	+++	+++
HNC-97	1.9±0.9	++	++	-	-	+	+
HNC-97	1.9±0.9	++	++	-	-	+	+
HNC-124	1.9±1.2	++	++	+	-	++	++
HNC-160	2.2±1.1	++	+	+	+	++	+
HNC-212	4.22±1.8	+++	+++	++	+++	++	+
HNC-212	4.22±1.8	+++	+++	++	+++	++	+
HNC-91	5.3±2.0	++	-	+	+++	+++	++
HNC-41	5.4±1.6	++	+	+	-	+++	+
HNC-206	8.3±2.4	+++	+++	+	+	+	++
HNC-136	9.3±1.9	++	-	-	-	+++	++
HNC-199	25±3.3	+	++	++	++	+++	+++
HNC-150	33±2.7	++	++	-	++	++	+

Figure 4. PTEN and ABCG2 expression in HNSCC cell lines. PTEN and ABCG-2 expression in HNSCC cellular extracts was analysed by Western blotting, utilising ß-actin as the internal standard.

expression varied among cell lines, with higher levels of the former in HCN-211 and of the latter, in HCN-212 and -91 (Fig. 3). The tumour suppressor PTEN, was expressed ubiquitously in all cell lines (Fig. 4). Recent evidence of a relationship between gefitinib and ABCG2 prompted us to characterise this drug efflux pump in our *in vitro* model; it was expressed in all cell lines with a higher expression in HNC-180, -199 and -136 (Fig. 4). The results of the various cellular targets analysed are summarised in Table IV.

Finally, mutational analysis, performed in the region of the EGFR gene coding for the tyrosine kinase domain and in the ATPase domain, showed that all cell lines were wild-type. However, this characterisation, utilising primers designed for ~150 nucleotides downstream and upstream of the exonic region, evidenced intronic variants. HCN-211 presented IVS17-104 C>A and IVS18+103 C>T intronic variants while HCN-41 cell line only IVS17-104 C>A. Intron 19 presented two different types of intronic variants: IVS19+96 A>G in HCN-91 and HCN-211, and IVS 19-60 in HCN-41, -91, -199 and -211. However, these intronic variants did not appear to interfere with the modification of EGFR expression.

Discussion

The validity of our *in vitro* model for a complete analysis of gefitinib activity in HNSCC is supported by the evaluation of gefitinib efficacy in this panel, characterised by significant cell line-specific drug variability, i.e. HNC-211, was highly sensitive (IC$_{50}$ in nanomolar range) while HNC-150 showed the highest resistance (IC$_{50}$=33 μM). Moreover, gefitinib was able to induce apoptosis after 3 days of exposure mainly in the most resistant cell lines. This was probably due to the high drug concentration responsive to a non-specificity of action. Notably, this TK inhibitor also induced apoptosis in two sensitive cell lines, suggesting that its ability to initiate the apoptotic process could be cell line-specific. This idea is supported by the evidence that in the resistant cells, apoptosis increased in a time-dependent manner leading to the hypothesis that the mechanism of drug saturation could be involved. Analysis of the modulation of EGFR signal transduction pathways by gefitinib showed that this drug, after three days of exposure, did not seem to inhibit EGFR phosphorylation. However, based on our previous evidence (40), we hypothesised that gefitinib inhibited EGFR activation early on, while after a prolonged exposure (3 days), the phosphorylated form of the receptor recovered its baseline level. Analysis of the ability of gefitinib to reduce the activation of the two main signal transduction pathways, PI3K/Akt/mTor and Ras/MAPK, was evidenced by a selective action on the first of these targets. Furthermore, PTEN was similarly expressed in all cell lines, in agreement with a low percentage of the inactivated form of this tumour suppressor reported in head and neck cancer (33,41), and the ubiquitous expression of ABCG2 did not allow the hypothesis of any correlation of this drug efflux pump with gefitinib sensitivity.

The cell variability for drug sensitivity suggested that we characterise our *in vitro* panel of HNSCC cell lines searching for predictive factors for gefitinib efficacy. We considered all biological characteristics involved in head and neck

tumour progression, such as HPV infection, ploidia, Fhit characteristics and p53 family expression. High-risk HPVs have been frequently found in HNSCC (30,31) but unfortunately, none of the 12 HNSCC cell lines showed the presence of HPV DNA (types 4, 5, 8, 9, 12, 14, 15, 17, 19-24, 26, 36-38, 47-50, 60 and 65). Moreover, only two cell lines, with medium sensitivity to gefitinib, were positive for Fhit expression suggesting that this factor is not directly correlated with the activity of this drug. The analysis of p53 family expression showed a variable distribution of cell lines with mutated p53 and/or the presence of the various isoforms of p63 and p73. No dependency on the drug activity was shown by the status of the p53 family. All cell lines were aneuploid with no difference between the most and the least sensitive lines. The lack of a dependency of gefitinib efficacy on the above described factors involved in head and neck cancer progression, prompted us to investigate the existence of a correlation between the activity of this drug and the expression level of cellular targets, such as TK receptors, downstream effectors, PTEN, and ABCG2, a drug efflux pump recently linked with gefitinib response (36-39). As reported in Table IV, the different distribution of the total and active forms of these targets did not allow the identification of any predictive factors, confirming that, as in other tumour types, gefitinib effectiveness in head and neck tumours, is not directly correlated with its target, EGFR. Finally, based on evidence that in lung cancer, certain mutations in the region of the EGFR gene coding for the ATPase and tyrosine kinase domains are related to drug sensitivity, we analysed our 12 cell lines for EGFR mutation status, confirming the data of Puhringer-Oppermann *et al* which suggested that these mutations are not relevant in determining gefitinib response in oral cancer (42).

Although our cell characterisation did not identify any valid predictive factor for gefitinib effectiveness, the comparison between p53 family and EGFR expression suggested, for the first time, a correlation between the ΔNp63 isoform and EGFR expression level.

Transcription factors of the p53 family are mainly considered to be tumour suppressors, even though recent evidence demonstrated opposite functions for the p63 isoforms, ΔNp63 and TAp63, promoting cell proliferation and mimicking tumour suppressor p53 respectively (27). Moreover, p53 family transcription factors are involved in the regulation of EGFR expression (23,24). In our study, two of the HNSCC cell lines did not express either ΔNp63 or EGFR, while the remainder co-expressed both proteins, suggesting a direct correlation between EGFR and ΔNp63 expression in the development of head and neck squamous cell carcinomas.

In conclusion, our study, performed in a broad panel of HNSCC cell lines, strongly confirmed the validity of gefitinib utilisation in head and neck cancer, however, it also demonstrated an urgent need to identify some valid predictive factors for the utilisation of tyrosine kinase inhibitors.

Acknowledgements

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