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Mitogen-activated Protein Kinase Pathway Is Activated in Oral Cancer Cell Lines Overexpressing Epidermal Growth Factor Receptor

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The receptor tyrosine kinase-coupled mitogen-activated protein kinase (MAP kinase) pathway is a conserved eukaryotic signaling module and is extensively used for transcytoplasmic signaling to the nucleus. In human tumors, abnormal receptor signaling has been observed in some forms of neoplastic development. In order to investigate the correlation of EGFR expression with regulation for transcytoplasmic signaling to the nucleus in carcinogenesis progression, we determined the levels of EGFR, c-H-*ras*, c-*raf*, c-*myc*, c-*fos* and c-*jun* messages, and the activities of Raf kinase, MEK kinase and ERK/MAP kinase in normal, immortalized, and tumorigenic oral keratinocytes. The level of EGFR messages notably increased in a multistep fashion when normal cells were progressively transformed to tumor cells and the activities of Raf kinase, MEK and ERK/MAP kinases in two tumor cell lines were enhanced compared to normal and immortalized cells. The activation of the Raf/MEK/ERK family of MAP kinases was closely associated with elevated expression of nuclear protein-encoded genes, c-*myc* and c-*jun*, in the two tumor cell lines. Taken together, MAP kinase pathway in signal transduction cascade is activated in oral cancer cell lines that overexpress EGFR.

Key words : signal transduction, EGFR, MAP kinase pathway, nuclear protein-encoded genes

Introduction

Signal transduction pathways regulated by bioactive molecules such as growth factors, cytokines, hormones, neurotransmitters or cell adhesion molecules (Miyajima *et al.*, 1988). An extracellular signal interacts with plasma membrane receptor to determine the cell's responses accordingly. External signals induce activation of specific genes and/or DNA replication through receptors. However, there are many instances where the same receptor induces different cellular responses, indicating that the nature of the signaling pathway inside the target cell is equally important (Arai *et al.*, 1997).

Following ligand binding, receptor dimerization, and autophosphorylation, Src homology 2 (SH2) domain-containing proteins are recruited to phosphorylated tyrosine residues on the receptor. These SH2 domain-containing proteins include the p85 components of the phosphoinositide 3-kinase pathway, phosphoinositide-specific phospholipase C (PLC)-γ in the protein kinase C pathway, Src family kinases, and ras-GTPase activating protein (GAP), Shc, and Grb2 in the Ras pathway (Schlessinger, 1994). In addition, receptor kinases can activate the p91 Jak/STAT pathway (Fu and Zhang, 1993; Silvennoinen *et al.*, 1993; Sadowski *et al.*, 1993). Recruitment to phosphorylated tyrosine residues on receptors leads to activation of the signaling molecules by several mechanisms (Marshall, 1995).

Mitogen-activated protein kinase (MAP kinase) pathway is a conserved eukaryotic signaling module that converts receptor signals into a variety of outputs (Marshall, 1995). Three distinct MAP kinase pathways are known to extensively be used for transcytoplasmic signaling to the nucleus, where transcription of specific genes is induced through phosphorylation and activation of transcription factors in vertebrates (Hunter, 1995). In the Ras/Raf/MEK/ERK pathway, one example of what are generically termed MAP kinase pathways, Raf corresponds to MAP kinase kinase kinase (MAPKKK), MEK corresponds to MAP kinase kinase (MAPKK), ERK corresponds to MAP kinase

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(Marshall, 1995). Central to the activation of this pathway is the activation of Ras to the GTP form through the promotion of guanine nucleotide exchange on Ras. This occurs through the complex of the exchange factor Sos and the adaptor protein Grb2 being recruited to tyrosine-phosphorylated receptors or through Shc-Grb2-Sos complexes (Schlessinger, 1994). GTP-bound Ras binds to the N-terminus of Raf family members, which are MAPKKKs, thus bringing Raf to the membrane. At the membrane, an uncharacterized event occurs that activates Raf and subsequently results in Raf autophosphorylation. Activated Raf phosphorylates and activates the cytoplasmic MEK1/MEK2 MAPKKs, which in turn phosphorylate and activate ERK1/ERK2. A fraction of the activated ERK1/ERK2 population translocates in the nucleus and phosphorylates transcription factors (Hill and Treisman, 1995). Activated ERK1/ERK2 can also phosphorylate cytoplasmic substrates, such as cytosolic phospholipase A2, which themselves can enter the nucleus and phosphorylate transcription factors (Hunter, 1995). In vertebrates the best-understood MAP kinase pathway leads to activation of the MEK1/MEK2 MAP kinases.

Recent studies have indicated that epidermal growth factor receptor (EGFR) is of fundamental importance in the regulation of epithelial proliferation and differentiation (Miettinen *et al.*, 1995; Sibilia and Wagner, 1995; Threadgill *et al.*, 1995). In human tumors, abnormal receptor signaling results in the overexpression of receptor or ligand or truncated forms of receptor, which are observed in some forms of neoplastic development (Gullick, 1991). Abnormal receptor signaling may thus, in part, be responsible for uncontrolled proliferation of cancer cells. Though detailed mechanisms for the abnormal receptor signaling of cancer cells remain unknown, abnormal receptor signaling may be due to the increased expression of EGFR and/or activation of the EGFR-mediated signal transduction cascade from cancer cells. To investigate this possibility, we determined i) the expression of EGFR and several genes associated with EGFR-mediated signal transduction cascade, ii) the activities of Raf kinase, MEK kinase, and ERK/MAP kinase, and iii) the expression of nuclear protein-encoded genes, c-*myc*, c-*fos* and c-*jun*, and cell cycle-associated p53 and *WAF1/CIP1* in primary normal human oral keratinocytes (NHOK), human papillomavirus (HPV)-immortalized human oral keratinocytes (HOK-16B), and two tumor cell lines derived from HOK-16B (CTHOK-16B-BaP and CTHOK-16B-DMBA).

Materials and Methods

Cell cultures

Primary NHOK were prepared from gingival tissue specimens and cultured in keratinocyte growth medium supplemented with pituitary extract (KGM; Clonetics, San Diego, CA) as previously described (Min *et al.*, 1995). The HOK-16B line that was immortalized by transfection of NHOK with cloned HPV-16 genome (Park *et al.*, 1991), was cultured in KGM. This line contained ~25 copies of intact HPV-16 DNA as integrated form per cell, expressed several viral specific poly(A^+) RNAs, and demonstrated immortality. Two tumor cell lines CTHOK-16B-BaP and CTHOK-16B-DMBA that were tumorigenic transformed by chronic exposure of the HOK-16B cells to benzo(*a*)pyrene and 7,12-dimethylbenz(a)-anthracene, respectively (Min *et al.*, 1995), were also cultured in KGM to eliminate any different cellular responses due to different culture media.

Northern blot hybridization analysis

To determine the cellular levels of EGFR, c-H-*ras*, c-*raf*, c-*myc*, c-*fos*, c-*jun*, p53, *WAF1/CIP1* and β-actin transcripts, cytoplasmic poly(A^+) RNA was extracted from cells using standard procedures. The obtained RNAs were resolved in a 1.2% agarose-formaldehyde gel and transferred onto Hybond-N (Amersham Corp., Arlington Heights, IL). Probes used for Northern analysis were synthesized using such templates as human EGFR cDNA (ATCC, Rockville, MD), v-H-*ras* cDNA (ATCC), v-*myc* cDNA (ATCC), v-*fos* cDNA (ATCC), v-*jun* cDNA (ATCC), p53 cDNA (from Dr E. Harlow, Massachusetts General Hospital Cancer Center, Charlestown, MA), *WAF1/CIP1* cDNA (from Dr B. Vogelstein, Johns Hopkins University, Baltimore, MD), and β-actin cDNA (from Dr. L. Kedes, Stanford University, Palo Alto, CA). The probes were labeled with [^{32}P]dCTP (Amersham Corp.) by megaprime labeling kit (Amersham Corp.). Hybridization was done as recommended by the membrane manufacturer. Filters were then autoradiographed on Hyperfilm-MP (Amersham Corp.) for 12 h at −70°C. After exposure, the probe was stripped off the filter for rehybridization to the next radiolabeled probe.

Western analysis

Eighty percent confluent cells were lysed, and the cell extracts were processed for Western analysis to determine the intracellular levels of p53, $p21^{WAF1/CIP1}$, Raf-1, MEK-1, and ERK1/ERK2 proteins using Western-Light kit (Tropix, Inc., Bedford, MA) as described previously (Min *et al.*, 1995). Monoclonal antibody to p53 (PAb 1801) was obtained from Oncogene Sciences (Uniondale, NY) and monoclonal antibodies against $p21^{WAF1/CIP1}$ (187), MEK-1 (H-8), and ERK2 (D-2) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibody to Raf-1 (C-12) was also purchased from Santa Cruz Biotechnology, Inc. After probing with the respective antibodies, the membrane was stained with 1X Ponceau S stain for 10 min to reveal total protein amount that was loaded per lane.

Determination of Raf kinase, MEK kinase, and ERK/MAP kinase activities

Cells were lysed with kinase-lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM EDTA, and 1% Triton X-100. Three hundred μg proteins in the cell lysates were incubated with the anti-human Raf-1 (C-12) polyclonal antibody (Santa Cruz Biotechnology, Inc.), anti-human MEK-1 (H-8) monoclonal antibody (Santa Cruz Biotechnology, Inc.) or anti-human ERK2 (D-2) monoclonal antibody (Santa Cruz Biotechnology, Inc.), and further incubated with Protein G agarose (Oncogene Sciences). The immunocomplexes were washed with RIPA buffer and resuspended in 12 μl of RIPA buffer. The activities of Raf kinase, MEK kinase, and ERK/MAP kinase were determined using MAP Kinase assay kit (Stratagene, La Jolla, CA) under the conditions recommended by the manufacturer. MEK-1 (Santa Cruz Biotechnology, Inc.), MAP kinase p42 (Santa Cruz Biotechnology, Inc.), and PHAS-I (Stratagene) were used as a substrate to quantitatively determine Raf kinase, MEK kinase, and ERK/MAP kinase activities, respectively. After incubation at 37°C for 30 min, the reaction was stopped by the addition of 2X SDS-PAGE sample buffer to the incubation mixture. Proteins were separated on a 10% polyacrylamide-SDS gel and the gel was dried and band was detected by exposing to Hyperfilm-MP (Amersham Corp.) at −70°C.

Results

Malignant conversion of normal oral keratinocytes increases the EGFR, c-myc, and c-jun mRNA levels

To examine whether expression of EGFR, c-H-*ras*, c-*raf*, c-*myc*, c-*fos*, and c-*jun* is dysregulated during malignant conversion of normal epithelial cells, we investigated the mRNA levels of these genes in normal, immortalized, and two tumor cell lines derived from the immortalized cells. As shown in Fig. 1A, EGFR mRNA level notably increased from normal to HPV-immortalized to tumor cells. Level of EGFR transcripts in the immortalized and tumorigenic oral keratinocytes was higher than that of normal cells. Similarly, c-*myc* and c-*jun* mRNA levels in the immortalized and in the tumor cell lines was notably increased compared to normal cells (Fig. 1D and F). Interestingly, c-*fos* mRNA level was high in normal, immortalized, and one of the tumor cell lines, CTHOK-16B-DMBA, but notably reduced in the CTHOK-16B-BaP cells (Fig. 1E). Though all of the

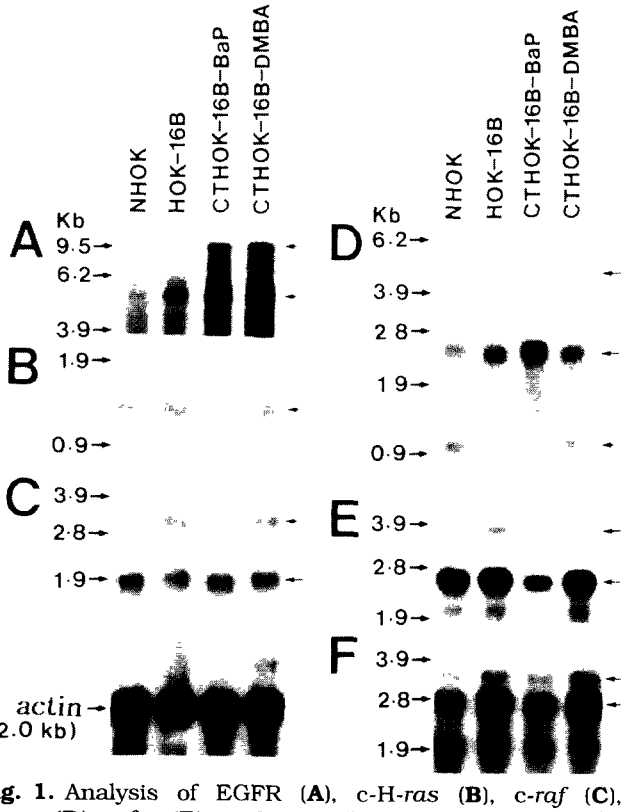

Fig. 1. Analysis of EGFR (**A**), c-H-*ras* (**B**), c-*raf* (**C**), c-*myc* (**D**), c-*fos* (**E**), and c-*jun* (**F**) transcripts. Poly(A^+)-RNAs extracted from NHOK, HOK-16B, CTHOK-16B-BaP, and CTHOK-16B-DMBA cells were resolved in a 1.2% agarose-formaldehyde gel and transferred onto nylon filter. Signals were detected using probes representing human EGFR, v-H-*ras*, v-*raf*, v-*myc*, v-*fos*, v-*jun* and β-actin cDNAs. Arrows indicate the position of the transcripts.

tested cells expressed c-H-*ras* and c-*raf* messages, mRNA levels of these genes were similar during malignant conversion of normal oral keratinocytes (Fig. 1B and C).

Malignant conversion of normal oral keratinocytes increases the protein levels of Raf-1, MEK-1 and ERK1/ERK2, and their kinase activities

To find out whether expression of Raf-1, MEK-1, and ERK1/ERK2 proteins is dysregulated when normal epithelial cells were progressively transformed to tumor cells, we determined the protein levels of these genes from the normal, immortalized, and tumorigenic cells. All of the tested cells expressed Raf-1, MEK-1, and ERK1/ERK2 proteins. The level of Raf-1 protein (74 kDa) was low in normal cells but increased notably in the immortalized and tumorigenic cells (Fig. 2A). Similarly, the protein levels of MEK-1 (42 kDa) and ERK1/ERK2 (44/42 kDa) in one of the tumor cell lines, CTHOK-16B-DMBA, were also notably higher than that in normal cells. However, such changes were not observed from the HPV-immortalized HOK-16B and one of the tumor cell lines CTHOK-16B-BaP (Fig. 2B and C). Particularly, the level of MEK-1 protein in these cell lines was somewhat lower than that in normal cells. Since alteration in Raf-1, MEK-1, and ERK1/ERK2 protein expression was observed, we further investigated their kinase activities from normal, immortalized, and tumorigenic oral keratinocytes. We studied the activities of Raf kinase, MEK kinase, and ERK/MAP kinase by immunoprecipitating cell lysates with Raf-1, MEK-1, and ERK2 antibodies as described in Materials and Methods. The activity of Raf kinase was low in normal cells but steadily enhanced when normal cells were progressively transformed to tumor cells (Fig. 3A). MEK kinase and ERK/MAP kinase activities in the tumor cell lines were similar, but notably higher than those in normal and immortalized cells (Fig. 3B and C). ERK/MAP kinase activity in normal cells was notably higher

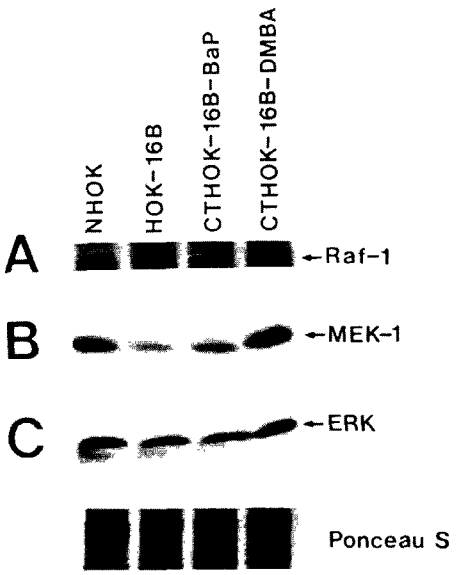

Fig. 2. Western blot analysis for the intracellular Raf-1 (**A**), MEK-1 (**B**), and ERK1/ERK2 (**C**) levels in NHOK, HPV-immortalized HOK-16B line, and two tumor cell lines derived from HOK-16B (CTHOK-16B-BaP and CTHOK-16B-DMBA). The membrane was stained with Ponceau S stain to reveal the total protein loaded per each lane. A structural protein stained with Ponceau S serves as the internal control to account for the loading error (lower panel).

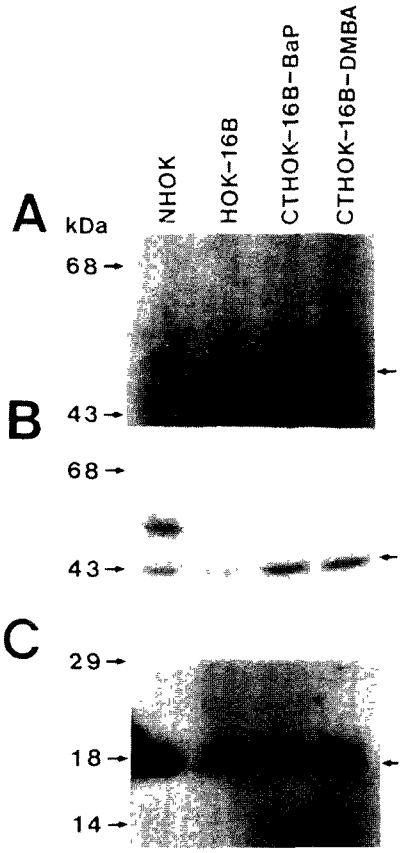

Fig. 3. Activities of Raf kinase (**A**), MEK kinase (**B**), and ERK/MAP kinase (**C**) in NHOK, HPV-immortalized cells, and two tumor cell lines CTHOK-16B-BaP and CTHOK-16B-DMBA. Autoradiogram of the MEK-1, MAP kinase p42, and PHAS-I proteins incubated with anti-Raf-1, anti-MEK-1, and anti-ERK2 immunoprecipitates, respectively. The phosphorylated proteins are marked with arrows. Activities of Raf kinase, MEK kinase, and ERK/MAP kinase in cell lysates were determined as described in "Materials and Methods".

than those in the other cells in this particular figure (Fig. 3C). These results indicate that the Ras/Raf/MEK/ERK pathway is activated from tumorigenic cells.

Malignant conversion of normal oral keratinocytes decreases the levels of p53 and p21$^{WAF1/CIP1}$ proteins but not their mRNA levels

The level of p53 transcript in the immortalized HOK-16 cells was notably higher than that of normal cells as previously reported (Min *et al.*, 1995). Higher level of p53 messages was also noticed in the both CTHOK-16B-BaP and CTHOK-16B-DMBA cells (Fig. 4A). The amount of p53 protein was significantly (1/3-fold) decreased in the immortalized and tumorigenic cells compared to that in normal cells (Fig. 4C). To find whether the diminished p53 level is linked to lower p53 function, we determined the p21$^{WAF1/CIP1}$ level from these cells. The immortalized and tumorigenic cells contained notably lower level of p21$^{WAF1/CIP1}$ as did normal cells (Fig. 4D). Although the level of p21$^{WAF1/CIP1}$ was notably decreased in the immortalized and CTHOK-16B-DMBA cells, the *WAP1/CIP1* transcript levels in these cells were similar to each other when densitometrically analyzed after normalization by β-actin messages. The amount of this gene transcription, however, was notably lower in the CTHOK-16B-BaP cells than other cells (Fig. 4B). Thus, the amount of *WAP1/CIP1* message was dependent upon chemical carcinogens used in the cell transformation in the HOK-16B cells.

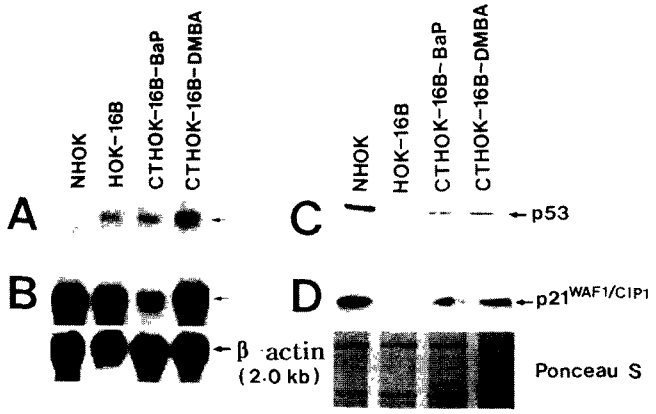

Fig. 4. Levels of p53 and *WAP1/CIP1* transcripts and proteins in NHOK, HPV-immortalized cells, and two tumor cell lines (CTHOK-16B-BaP and CTHOK-16B-DMBA). Northern blot analysis of poly(A$^+$)RNAs to ^{32}P-labeled p53 (**A**) and WAP1/CIP1 (**B**) cDNAs. Western blot analysis of electrophoretically separated p53 (**C**) and *WAP1/CIP1* (**D**) proteins. The membrane was stained with Ponceau S stain to reveal the total protein loaded per each lane. A structural protein stained with Ponceau S serves as the internal control to account for the loading error (lower panel).

Discussion

We previously established and characterized an HPV-immortalized HOK-16B line (Park *et al.*, 1991) and two tumor cell lines derived from HOK-16B, CTHOK-16B-BaP and CTHOK-16B-DMBA (Min *et al.*, 1995). Since the overexpression of EGFR is frequently found in human squamous cell carcinoma cell lines, we investigated the correlation of EGFR expression with regulation for transcytoplasmic signaling in carcinogenesis progression by determining the levels of EGFR, c-H-*ras* and c-*raf* messages, and the activities of Raf kinase, MEK kinase and ERK/MAP kinase in normal, immortalized, and tumorigenic oral keratinocytes. Our data show that the level of EGFR message notably increases in a multistep fashion when the normal cells were progressively transformed to tumor cells, whereas c-H-*ras* and c-*raf* messages of the tested cells were similar to each cell type. Also, the activities of Raf kinase, MEK kinase and ERK/MAP kinase in two tumor cell lines were notably enhanced compared to normal and immortalized cells. Although our study does not provide direct evidence, the presented data support that the overexpression of EGFR is necessary for the up-regulation of the kinase activities of MAP kinase pathway in signal transduction cascade of oral cancer cells and is also required for the conversion of normal cells to malignant phenotypes. Since various cell growth factors such as EGF and transforming growth factor-α promote cell proliferation through binding to EGFR (Massague, 1983), the overexpression of EGFR in cancer cells could result in a growth advantage. There is much evidence to support the involvement of EGFR overexpression at the mRNA level (Wong and Biswas, 1986; Gullick, 1991; Min *et al.*, 1995) and at the protein level (Shin *et al.*, 1994) in the head and neck carcinogenesis, suggesting that EGFR expression may play an important role in transformation from normal through premalignant changes to squamous cell carcinoma. Furthermore, studies have shown that patients with tumors showing the overexpression of EGFR have a

poor prognosis (Sainsbury *et al.*, 1987; Nicholson *et al.*, 1988).

Since the activation of ERK family of MAP kinases is known as a critical event in signal transduction from receptor tyrosine kinases (Cowley *et al.*, 1994; Mansour *et al.*, 1994), the up-regulation of Raf-1, MEK-1 and ERK2 in cancer cells can result in a growth advantage. The activation of Raf by receptor tyrosine kinases requires $p21^{Ras}$. The role of $p21^{Ras}$ is to recruit Raf to the plasma membrane (Leevers *et al.*, 1994; Stokoe *et al.*, 1994), where another tyrosine kinase-generated signal fully activates the membrane-bound Raf (Leevers *et al.*, 1994; Fabian *et al.*, 1994). In the receptor tyrosine kinase-coupled Ras/Raf/MEK/ERK pathway, activated Raf phosphorylates and activates a MEK, which in turn phosphorylates and activates another serine/threonine protein kinase ERK. These pathways serve to link signals from the cell surface to cytoplasmic and nuclear events. In addition, MAP kinase pathways mediate many cellular responses in mammalian cells (Freshney *et al.*, 1994; Han *et al.*, 1994; Rouse *et al.*, 1994). The association of activation of ERK pathway with carcinogenesis is supported by previous observations showing that expression of constitutively activated forms of MEK, generated by site-directed mutagenesis, induces mitogenesis and transformation in fibroblasts (Cowley *et al.*, 1994; Mansour *et al.*, 1994).

An early event in most oral carcinogenesis appears to be p53 dysfunction, i.e. absence, decreased level, or mutations (Min *et al.*, 1994). Wild type p53 is involved in the control of cell cycle progression, DNA repair and DNA replication presumably via the expression of *gadd*45 and $p21^{WAF1/CIP1}$ proteins (El-Deiry *et al.*, 1994). Decreased levels of wild type p53 results in the down-regulation of *gadd*45 and $p21^{WAF1/CIP1}$, which inhibits the activity of cyclin-dependent kinases (El-Deiry *et al.*, 1993). Our results show that the immortalized and tumorigenic cells contained lower level of p53 protein compared with that of normal cells. The low level of p53 proteins in these cells harboring HPV-16 DNA might result from the enhanced degradation of the protein by viral E6 protein (Min *et al.*, 1995). Since nuclear protein-encoded genes might be an important contributing factor to the proliferation properties of epithelial cells and might be a major target of signal transduction pathway, the c-*myc*, c-*fos* and c-*jun* mRNA levels were studied. The levels of c-*fos* transcripts were similar during the neoplastic conversion of oral keratinocytes but c-*myc* and c-*jun* mRNA levels were notably enhanced. Though further studies are required to delineate the detailed mechanisms of the uncontrolled proliferation of cancer cells, the conversion of NHOK to tumorigenic cells may, in part, be due to the up-regulation of receptor tyrosine kinase-coupled Ras/Raf/MEK/ERK pathway through overexpression of EGFR. It also demonstrates that the activation of the Raf/MEK/ERK family of MAP kinases is closely associated with elevated expression of nuclear protein-encoded genes, c-*myc* and c-*jun*, in tumorigenic oral keratinocytes.

Acknowledgements

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References

Arai, K., Tsuruta, L., Watanabe, S. and Arai, N.: Cytokine signal networks and new era in biomedical research. Mol. Cells 7: 1-12, 1997.

Cowley, S., Paterson, H., Kemp, P. and Marshall, C.J.: Activation of MAP kinase is necessary and sufficient for PC 12 differentiation and for transformation of NIH 3T3 cells. Cell 77: 841-852, 1994.

El-Deiry, W.S., Harper, J.W., O'Connor, P.M., Velvulescu, V.E., Canman, C.M., Jackman, J., Pietenpol, J.A., Burrell, M., Hill, D.E., Wang, D.E., Wiman, K.G., Mercer, W.E., Kastan, M.B., Kohn, K. W., Elledge, S.J., Kinzler, K.W. and Vogelstein, B.: WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res. 54: 1169-1174, 1994.

El-Deiry, W.S., Tokino, T., Velvulescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W. and Vogelstein, B.: WAP1, a potential mediator of p53 tumor suppressor. Cell 75: 817-825, 1993.

Fabian, J.R., Vojtek, A.B., Cooper, J.A. and Morrison, D.K.: A single amino acid change in Raf-1 inhibits Ras binding and alters Raf-1 function. Proc. Natl. Acad. Sci. USA 91: 5982-5986, 1994.

Freshney, N.W., Rawlinson, L., Guesdon, F., Jones, E., Cowley, S., Hsuan, J. and Saklatvala, J.: Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of hsp27. Cell 78: 1039-1049, 1994.

Fu, X.-Y. and Zhang, J.-J.: Transcription factor p91

interacts with the epidermal growth factor receptor and mediates activation of the c-*fos* gene promoter. Cell 74: 1135-1145, 1993.

Gullick, W.J.: Prevalence of aberrant expression of the epidermal growth-factor receptor in human cancers. Br. Med. Bull. 47: 87-98, 1991.

Han, J., Lee, J.-D., Bibbs, L. and Ulevitch, R.J.: A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science 256: 808-811, 1994.

Hill, C.S. and Treisman, R.: Transcriptional regulation by extracellular signals: mechanism and specificity. Cell 80: 199-211, 1995.

Hunter, T.: Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. Cell 80: 225-236, 1995.

Leevers, S.J., Paterson, H.F. and Marshall, C.J.: Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. Nature 369: 411-414, 1994.

Mansour, S.J., Matten, W.T., Hermann, A.S., Candia, J.M., Rong, S., Fukasawa, K., Vande Woude, G.F. and Ahn, N.G.: Transformation of mammalian cells by constitutively active MAP kinase. Science 265: 966-970, 1994.

Marshall, C.J.: Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80: 179-185, 1995.

Massague, J.: Epidermal growth factor-like transforming growth factor II: interaction with epidermal growth factor receptors in human placenta membranes and A431 cells. J. Biol. Chem. 258: 13614-13620, 1983.

Miettinen, P.J. Berger, J.E., Meneses, J., Phung, Y., Pederson, R.A., Werb, Z. and Derynck, R.: Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. Nature 376: 337-341, 1995.

Min, B.-M., Baek, J.H., Shin, K.H., Gujuluva, C.N., Cherrick, H.M. and Park, N.-H.: Inactivation of the p53 gene by either mutation or HPV infection is extremely frequent in human oral squamous cell carcinoma cell lines. Eur. J. Cancer. (Oral Oncology) 30B: 338-345, 1994.

Min, B.-M., Woo K.M., Baek, J.H., Lee, G. and Park, N.-H.: Malignant transformation of HPV-immortalized human oral keratinocytes by chemical carcinogens. Int. J. Oncol. 7: 249-256, 1995.

Miyajima, A., Miyatake, S., Schreurs, J., de Vries, J.E., Arai, N., Yokota, T. and Arai, K.: Coordinate regulation of immune and inflammatory responses by T cell-derived lymphokines. FASEB J. 2: 2462-2473, 1988.

Nicholson, S, Halcrow, P. Jr., Sainsbury C., Angus, B., Chambers, P., Farnon, J.R. and Harris, A.L.: Epidermal growth factor receptor (EGFR) status associated with failure of primary endocrine therapy in elderly postmenopausal patients with breast cancer. Br. J. Cancer 58: 810-814, 1988.

Park, N.-H., Min, B.-M., Li, S.-L., Huang, M.Z., Cherrick, H.M. and Doniger, J.: Immortalization of normal human oral keratinocytes with type 16 human papillomavirus. Carcinogenesis 12: 1627-1631, 1991.

Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T. and Nebreda, A.R.: A noble kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the smal heat shock proteins. Cell 78: 1027-1037, 1994.

Sadowski, H.B., Shuai, K., Darnell, J.E. and Gilman, M. Z.: A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. Science 261: 1739-1744, 1993.

Sainsbury, J.R.C., Farnon, J.R., Needham, G.K., Malcolm, A.J. and Harris A.L. : Epidermal growth factor receptor states as predictor of early recurrence of and death from breast cancer. Lancet i: 1398-1402, 1987.

Schlessinger, J.: SH2/SH3 signaling proteins. Curr. Opin. Genet. Dev. 4: 25-30, 1994.

Shin, D.M., Gimenez, I.B., Lee, J.S., Nishioka, K., Wargovich, M.J., Thacher, S., Lotan, R., Slaga, T.J. and Hong, W.K.: Expression of epidermal growth factor receptor, polyamine levels, ornithine decarboxylase activity, micro-nuclei, and transglutaminase I in a 7, 12-dimethylbenz(a)anthracene-induced hamster buccal pouch carcinogenesis model. Cancer Res. 50: 2505-2510, 1994.

Sibilia, M. and Wagner, E.F.: Strain-dependent epithelial defects in mice lacking the EGF receptor. Science 269: 234-238, 1995.

Silvennoinen, O., Schindler, C., Schlessinger, J. and Levy, D.E.: Ras-independent growth factor signaling by transcription factor tyrosine phosphorylation. Science 261: 1736-1739, 1993.

Stokoe, D., Macdonald, S.G., Cadwallader, K., Symons, M. and Hancock, J.F.: Activation of Raf as a result of recruitment to the plasma membrane. Science 264: 1463-1467, 1994.

Threadgill, D.W., Dlugosz, A.A., Hansen, L.A., Tennenbaum, T., Lichti, U., Yee, D., La-Mantia, C., Mourton, T., Herrup, K., Harris, R.C., Barnard, J.A., Yuspa, S.H., Coffey, R.J. and Magnuson, T.: Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. Science 269: 230-234, 1995.

Wong, D.T.W. and Biswas D.K.: Activation of c-erbB oncogene during DMBA-induced carcinogenesis in hamster cheek pouch. J. Dent. Res. 65: 221, 1986.