学　位　論　文

Doctor's Thesis

論文題名：**Activation of the extracellular signal-regulated kinase signaling pathway in squamous cell carcinoma of the skin**
（ヒト皮膚有棘細胞癌における細胞外シグナル制御キナーゼの活性化について）

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CONTENTS

Summary

Cutaneous squamous cell carcinoma (SCC) is the second most common skin cancer after basal cell carcinoma (BCC) and causes the majority of deaths among the non-melanoma skin malignancies. Although widespread and increasing in incidence, SCC has been poorly understood at the level of molecular pathogenesis until recently. Mitogen-activated protein kinases (MAPKs) are a widely conserved family of serine/threonine protein kinases involved in many cellular programs such as cell proliferation, differentiation, motility, and death. One important group of MAPKs is the extracellular signal-regulated kinase (ERK). This pathway is up-regulated in many types of human tumor. Recent studies revealed that activation of ERKs plays a critical role in the proliferation of cancer cells and is an attractive target for anti-cancer therapy. However, the role of activated ERK1/2 in SCC of the skin remains unknown. In this study, the expression and distribution of phosphorylated ERK1/2 (p-ERK1/2) in normal human skin and SCC with different degrees of differentiation was examined by immunohistochemical analysis using formalin-fixed paraffin embedded sections. PD98059, a specific ERK

pathway inhibitor, was used to evaluate the effect of blockade of ERK activation on the proliferation of cutaneous SCC cell line (DJM-1) in culture. The results indicate that Positive nuclear staining for activated ERK1/2 was observed in all cases of SCC examined in this study but rarely in the control specimens of normal skin. Moreover, the expression of activated ERK1/2 was found significantly higher in poorly differentiated SCC as compared with well- differentiated ones. The expression levels were positively associated with the degrees of malignancy and proliferative activities of SCC. On the other hand, the inhibition of ERK signaling pathway markedly suppressed tumor cell proliferation. These results suggest that ERK1/2 signal pathways play an important role in the proliferation of SCC and that the inhibition of this signal pathway may be effective in the treatment of cutaneous SCC.

Summary in Japanese

[背景と目的]

研究対象の有棘細胞癌（squamous cell carcinoma:SCC）は、基底細胞癌（basal cell carcinoma:BCC）と併せて non-melanoma skin carcinoma (NMSC)と総称される。欧米白人では NMSC はあらゆる悪性腫瘍の中で最も高頻度に見られるものであり、全悪性腫瘍の半数以上を占める。日本の統計でも、全国の代表的な 100 施設において年間約 700 例程度が登録され、約 1000 の BCC に次いで 2 番目に多い。SCC の早期病変である日光角化症 800 例と併せると、皮膚悪性腫瘍中、最も多い数字となる。さらに、近年の人口の高齢化および日光暴露機会の増加と言った生活習慣の変化を反映して、SCC の発症数は年々増加傾向にあり、SCC の発癌機構の解明や診断と治療法の開発は現代医療の急務となっているが、その分子レベルでのメカニズムは、現在に至るまでほとんど分かっていない。

マイトジェン活性化タンパク質キナーゼ（MAPK）ファミリーは重要な様々の機能を担っている．特に細胞外シグナル制御キナーゼ 1 および 2 (ERK1/2) の活性化は，細胞の増殖や分化に関わる。また、

種々の悪性腫瘍において、ERK1/2 キナーゼが恒常的に活性化されている症例が高頻度に見出され、その発症や増殖のメカニズムに関与していることが示唆されている。本研究では、正常皮膚および皮膚有棘細胞癌組織における活性化 ERK1/2 キナーゼの発現およびヒト皮膚有棘細胞癌由来株（DJM-1）における ERK－MAPK キナーゼｶｽｹｰﾄﾞの選択的遮断による増殖抑制効果について検討することを目的とした。

[方法]

1：免疫組織化学的解析

皮膚有棘細胞癌（高分化型１０例、低分化型１０例）と健常人皮膚５例の手術標本のパラファンブロックから切片を作製した。活性化 ERK1/2 を検出する抗体を用いて avidin-biotin complex(ABC) 方法による免疫組織化学的検討を行った。

２：細胞増殖抑制効果の検討

皮膚有棘細胞癌由来細胞株(DJM-1)を２４穴プレートに播種して、各種濃度の ERK 阻害剤 PD98059 を添加し２４～７２時間培養した後、コールタカウンタにて細胞数を計測し、増殖への影響を評価した。

[結果]

1：正常皮膚における活性化 ERK1/2 の発現

正常皮膚では、ごく一部の汗管の管腔内面の細胞膜に線状に陽性反応があった。また少数の血管内皮細胞の核内にも極めて弱く陽性反応が観察された。すべての表皮と毛嚢細胞には活性化 ERK1/2 の発現が検出されなかった。

2：皮膚有棘細胞癌における活性化 ERK1/2 の発現

活性化 ERK1/2 蛋白は、高分化型有棘細胞癌では、散在性に角化傾向の乏しい腫瘍細胞の核にのみ、免疫陽性反応を示した。低分化型有棘細胞癌では、高分化型有棘細胞癌にくらべて、より多数の腫瘍細胞に陽性反応が認められた。腫瘍細胞の分化度が低くなる程、すなわち悪性度が高くなる程活性化 ERK1/2 の発現が増加し、両者間に統計学的な有意差がみとめられた。

活性化 ERK1/2 は、癌細胞のほか、腫瘍周囲の血管内皮細胞の核内にも強く発現していた。

３：PD98059 の細胞増殖抑制効果

DJM-1 細胞では、PD98059 は濃度依存性に細胞増殖を抑制し、30 μM で著明な阻害効果を認めた。

[考察]

免疫組織学的検討において、正常皮膚では、活性化 ERK の発現は非常に弱く少数の血管内皮と汗管の管腔内面の細胞膜に限られ、表皮や毛嚢には検出されなかった。一方、皮膚有棘細胞癌では、腫瘍細胞の増殖能に関連して強く発現しており、その発症や増殖のメカニズムに関与している可能性が考えられる。さらに DJM-1 細胞を用いた PD98059 による ERK1/2 活性の選択的阻害実験において、その増殖が著明に抑制されたことから、ERK1/2 の活性化はヒト皮膚有棘胞癌における増殖において重要な役割を担っていることが示唆された。

[結論]

皮膚有棘細胞癌に対して、ERK 経路を標的にした分子標的治療の可能性が示された。

Main publications

Authors

Xiaoyong Zhang, Takamitu Makino, Faith C. Muchewa, Tong Lin, Shoji Wakasugi, Kiyofumi Egawa, and Hironobu Ihn.

Title

Activation of the extracellular signal-regulated kinases signaling pathway in squamous cell carcinoma of the skin.

Journal

Bioscience Trends.

(in press)

Other Publications

1: Xiaoyong Zhang, Kiyofumi Egawa, Yong Xie, and Hironobu Ihn. The expression of SnoN in normal human skin and cutaneous keratinous neoplasms. ***Int J Dermatol.*** (in press)

2: XIAOYONG ZHANG, TSUYOSHI ISHIHARA, TOMOMICHI ONO. Dermoid cyst at the suprasternal notch: an adult case. ***Scand J plast Reconstr Surg Hand Surg.*** 39: 57-59, 2005.

3: Yong Xie, Xiaoyong Zhang, Shoji Wakasugi, Takamitsu Makino, Yuji Inoue, and Hironobu Ihn. Immunohistochemical characterization of the cellular infiltrate in localized scleroderma. ***Int J Dermatol.*** (in press)

4: Yong Xie, Xiaoyong Zhang, Yuji Inoue, Shoji Wakasugi, Takamitsu Makino, and Hironobu Ihn. Expression of CD1a and CD86 on scleroderma Langerhans cells. ***Eur J Dermatol.*** (in press)

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Abbreviations

ATP:	adenosine trisphosphate
AML:	acute myeloid leukemias
BCC:	basal cell carcinoma
CRC:	colorectal cancer
DAB:	diaminobenzidine
DMSO:	dimethyl sulfoxide
EGFR:	epidermal growth factor receptor
ERK:	extracellular signal-regulated kinase
FCS:	fetal calf serum
JNK:	c-Jun N-terminal kinase
MAPK:	mitogen-activated protein kinase
MAPKK:	MAPK kinase
MAPKKK:	MAPK kinase kinase
MEK:	ERK kinase
MEM:	minimal essential medium
PD98059:	2'-Amino-3'-methoxyflavone
SCC:	squamous cell carcinoma

TGF- α: transforming growth factor α

U0126: 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophynyltio) butadiene

Chapter 1: Introduction

1.1 Cutaneous squamous cell carcinoma

Primary cutaneous squamous cell carcinoma (SCC) is a malignant tumor that arises from keratinizing cells of the epidermis or its appendages. It is locally invasive and has the potential to metastasize to other organs of the body [1]. These tumors occur predominantly on areas of the skin that have been exposed to the sun. Chronic exposure to sunlight appears to be more significant in the etiology of SCCs than in that of other skin tumors [2~4].

SCC is also strongly associated with advanced age, with a sharp increase in incidence after the age of 40 [5]. Today, the lifetime risk of SCC is approximately 15%, almost double that of two decades ago. Increased exposures to UV radiation (through increased use of tanning salons, more time spent outdoors, change in clothing style and ozone depletion) and increased longevity have been suggested as possible causes for the increase in incidence of SCC. It is likely that this trend will continue as a result of further depletion of the ozone layer and increasing age [6, 7].

SCC has significant potential for recurrence and metastasis [8, 9]. The factors associated with an increased risk of recurrence and metastases include tumor size, depth of tumor invasion, tumors arising in scars and degree of histologic differentiation. Tumor size has been shown to have a strong direct relationship with local recurrence rates, regional metastatic rates, and cure and survival rates [10]. SCCs greater than 2 cm in diameter have more than double the recurrence rate (15.7% vs. 5.8%) and triple the metastatic rate (23.4% vs. 7.6%) of lesions less than 2 cm in diameter [8]. The 5-year cure or survival rate for patients with tumors that are less than 2cm in diameter is considerably better than that for patients with lesions more than 2 cm in diameter (98.4 and 72.1% respectively) [10].

With respect to depth of tumor invasion, tumors greater than 4 mm in depth (excluding surface layers of keratin) or extending down to the subcutaneous tissue (Clark level V) are more likely to recur and metastasize (metastatic rate 45.7%) compared with thinner tumors [10~12]. Recurrence and metastases are less likely in tumors confined to the upper half of the dermis and less than 4 mm in depth (metastatic rate 6.7%) [10, 13].

SCC arising in sites of chronic trauma or scarring have a considerably higher metastatic rate than most other SCCs (Fig. 1). It is not certain whether this is due to some impairment in host response to the tumor or to the properties of the tumor itself, but many of these lesions are large, deep, and poorly differentiated [8]. Tumours arising in a chronic ulcer or sinus tract have an 18~31% metastatic rate, while those occurring in foci of scarring show metastases in 25-37.9% of cases [14, 15].

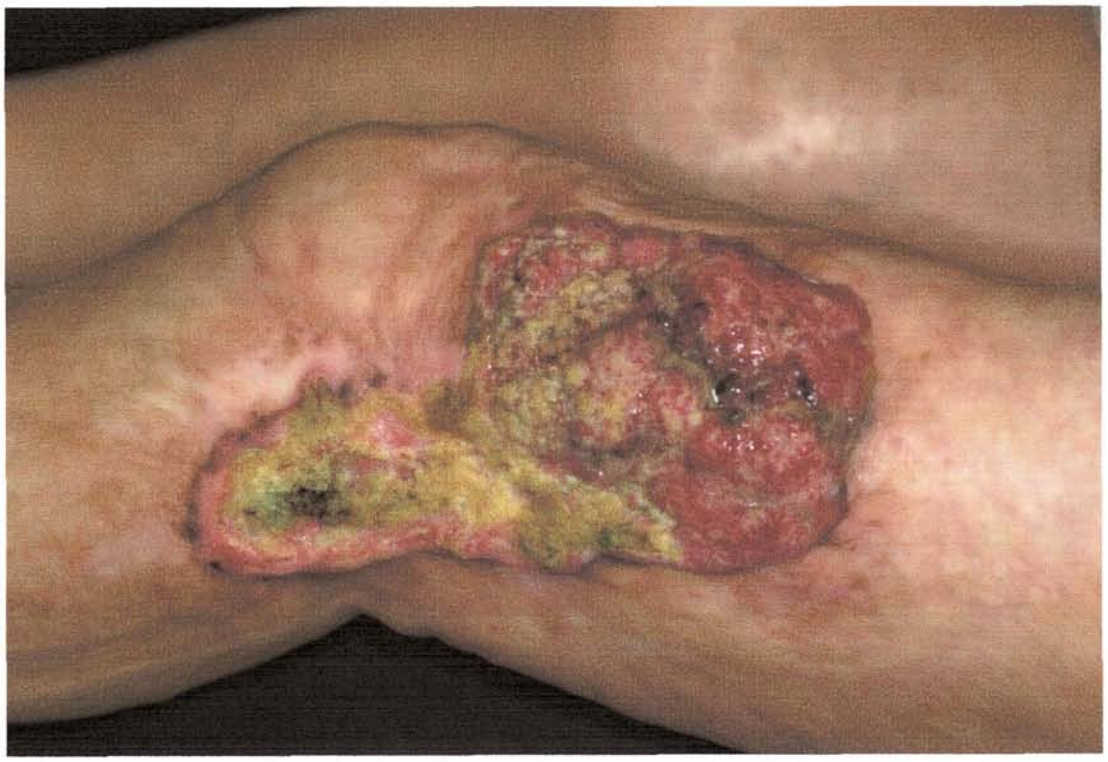

Fig.1: Clinical photo of squamous cell carcinoma of the skin: This large ulcerated tumour has arisen at the site of a previous burn.

Degree of histologic differentiation has an important effect on the probability of both local recurrence and metastatic risk, poorly differentiated SCCs (i.e. those of Broders' grades 3 and 4; Table 1) demonstrate a recurrence rate of 28.6% and metastatic rate of 32.8%, as compared to 13.6% and 9.2%, respectively, for well differentiated ones (i.e. those of Broders' grades 1 and 2) [1, 10, 16~19] (Fig. 2).

Table 1: Broders' histological classification of differentiation in cutaneous squamous cell carcinoma

Grade	% undifferentiated cell
1	< 25
2	< 50
3	< 75
4	>75

The histopathologic grade of differentiation in cutaneous squamous cell carcinoma was established by broders in 1932 [19].

SCC is the second most common skin cancer after basal cell carcinoma (BCC) and causes the majority of deaths among the non-melanoma skin malignancies [20~22]. Despite improvement in surgery, radiotherapy, and conventional chemotherapy, local recurrence following definitive

treatment is not uncommon, and metastasis and death may ensue. Once nodal metastasis of cutaneous SCC has occurred, the overall 5-year survival rate has historically been in the range of 25~35%. Prognosis is extremely poor for patients with a compromised immune system, with metastasis to multiple lymph nodes, or with cervical lymph nodes greater than 3 cm in diameter. Metastasis to distant organs remains incurable [10, 16, 23~25].

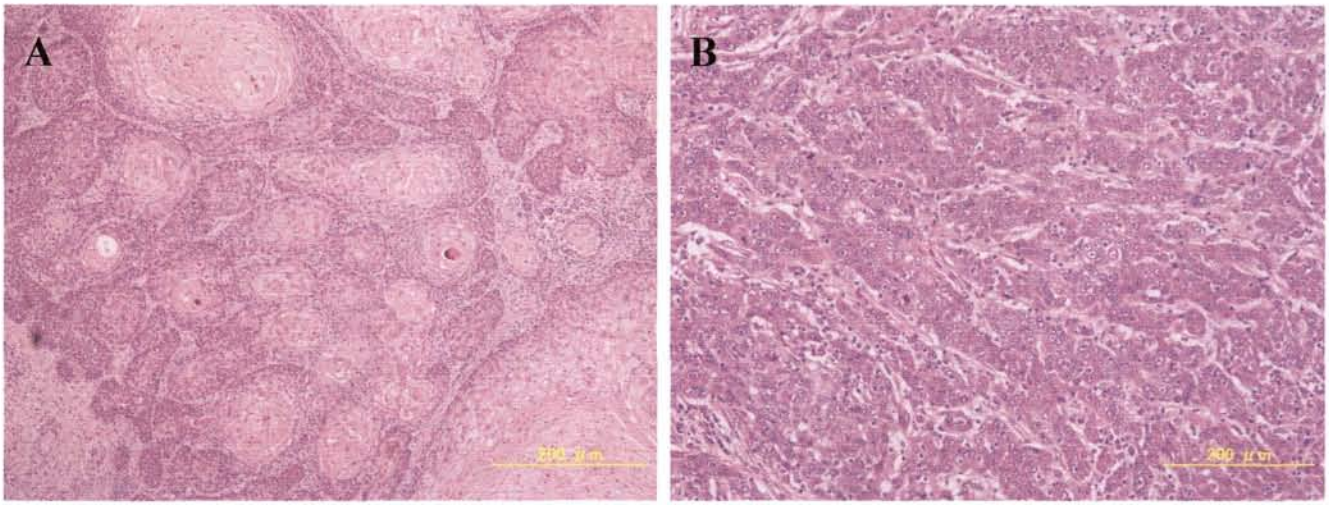

Fig.2: Histopathological photo of squamous cell carcinoma of the skin.

(A) Well-differentiated SCC: multiple irregular nests of mildly atypical squamous cells are seen with an inflamed stroma. Keratinization is present with some tumour nests.

(B) Poorly differentiated SCC: this tumour shows infiltrating strands and nests of moderately atypical cells. Intercellular bridges are not prominent, and no keratinization is seen. Mitotic activity is noted. (H&E stain)

In recent years, a new strategy has been evaluated for patients with cancer: targeted therapies that inhibit specific cancer pathways and molecules involved in tumor growth and progression. One of the most studied targets for anticancer therapy is the extracellular signal-regulated kinase (ERK) 1 and 2, a member of the Mitogen-activated protein kinase (MAPK) family of serine-threonine kinases.

Although widespread and increasing in incidence, SCC has been poorly understood at the level of molecular pathogenesis until recently. The role of activated ERK1/2 in cutaneous SCC has not been investigated.

1.2 Extracellular signal-regulated kinases

Protein kinases are enzymes that covalently attach phosphate to the side chain of either serine, threonine, or tyrosine of specific proteins inside cells. Such phosphorylation of proteins can control their enzymatic activity, their interaction with other proteins and molecules, their location in the cell, and their propensity for degradation by proteases. MAPKs compose a family of protein kinases whose function and regulation have been conserved during evolution from unicellular organisms such as

brewers' yeast to complex organisms including humans [26]. MAPKs phosphorylate specific serines and threonines of target protein substrates and regulate cellular activities ranging from gene expression, mitosis, movement, metabolism, and programmed death. Because of the many important cellular functions controlled by MAPKs, they have been studied extensively to define their roles in physiology and human disease.

Although each MAPK has unique characteristics, a number of features are shared by the MAPK pathways studied to date. The core of any MAPK pathway is composed of three tiers of sequentially activating protein kinases: a MAPK kinase kinase (MAPKKK), which activates a MAPK kinase (MAPKK), which in turn phosphorylates and activates a MAPK. An activated MAPK is then capable of entering the nucleus and regulating the activities of transcription factors or kinases further downstream by phosphorylation, and thereby controls gene expression and cellular function [27~29] (Fig. 3).

To date, at least six distinct groups of MAPKs have been characterized in mammals, of which three subfamilies of MAPK are the best understood. These are the extracellular signal-regulated kinases (ERK), the c-Jun

N-terminal kinases (JNK), and the p38 MAPK kinases (Fig. 4). In addition, other MAPK family members, including ERK3/4, ERK5, and ERK7/8 have been identified, but the biological role of these MAPKs is not well defined [30~33].

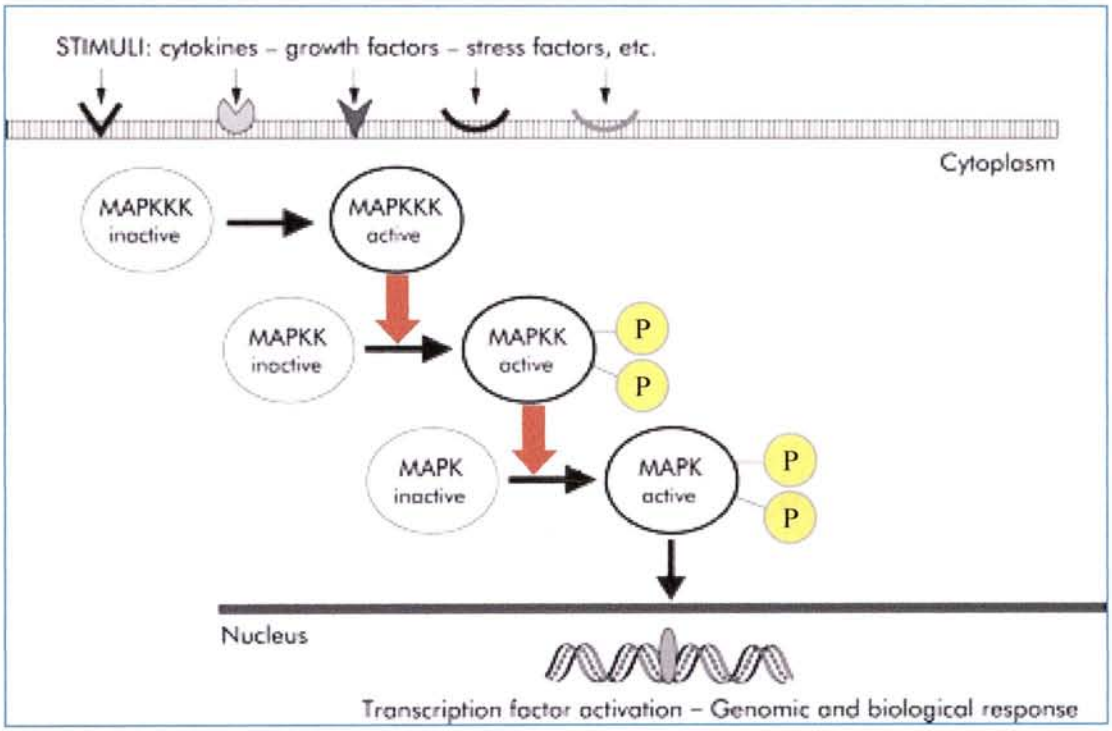

Fig. 3: MAP kinase signal transduction pathway

The MAPKs are a group of serine/threonine kinases that are activated in response to a diverse array of extracellular stimuli and mediate signal transduction from the cell surface to the nucleus. Extracellular stimuli lead to activation of a MAP kinase via a MAPK signaling cascade, which consists of three protrein kinases: MAPK, MAPKK and MAPKKK. Activated MAPKKK phosphorylates MAPKK and thus activated MAPKK in turn activates MAPK through phosphorylation. An activated MAPK is then capable of entering the nucleus and regulating the expression of target genes by phosphorylating relevant transcription factors.

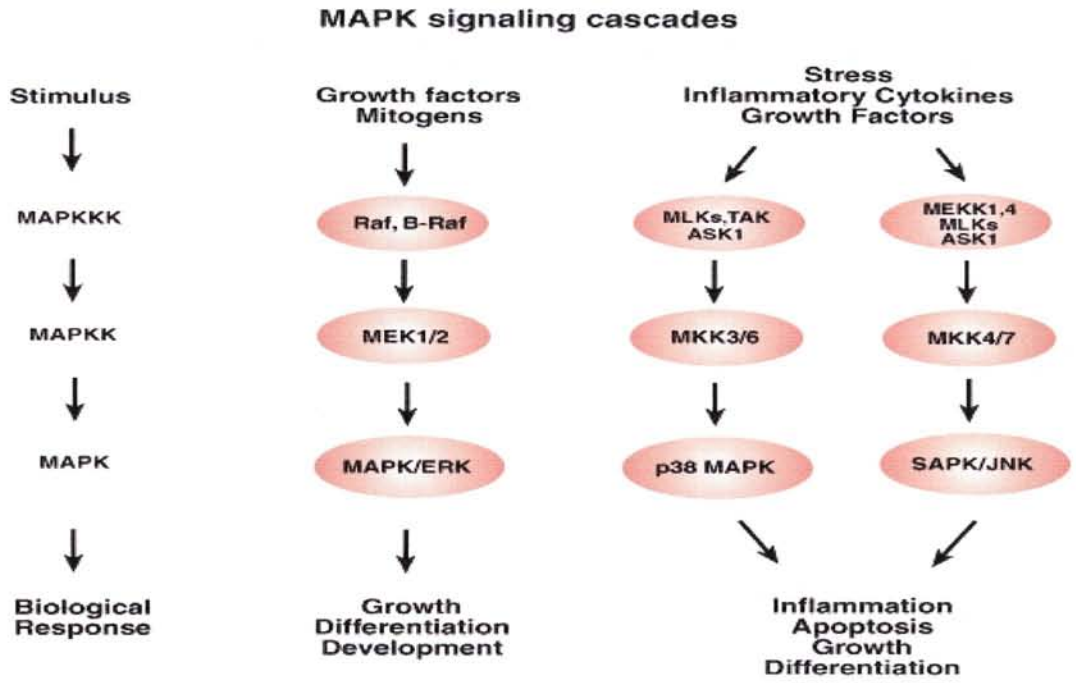

Fig. 4: MAP kinase families

The different members of the super-family of MAP kinases participate in signaling cascades conserved through evolution, which regulate important biologic activities. Three major groups of MAPKs exist: ERK1/2, JNKs- and p38 MAP kinase.

Each MAPK is activated through a specific phosphorylation cascade. The ERK are activated by most growth factors and have been shown to be a key regulator of both proliferation and differentiation in different cell types, while JNKs and p38 MAP kinase are activated by various cellular stresses and have predominantly been implicated in responses to cellular stress, inflammation and/or apoptosis [34].

On the other hand, three major MAP kinase pathways exist in human tissues, but the one involving ERK signaling pathway is most relevant to human cancer. A high level of p-ERK protein was frequently observed in several kinds of human tumors, including renal cell carcinoma, breast cancer, glial neoplasms, melanoma, prostate cancer and colorectal cancer [34~41]. Furthermore, recent advances in cancer research have shown that activation of ERK1/2 plays a critical role in the proliferation of cancer cells, and for this reason, this pathway has been the subject of intense research and pharmaceutical scrutiny to identify novel target-based approaches for cancer treatment [42~45]. In contrast, the role of ERK1/2 in cutaneous SCC was less clear. Therefore, the present study was performed to first examine the expression of p-ERK1/2 protein in cutaneous SCC specimens with different degrees of differentiation, and to clarify its correlation with SCC proliferation as potential therapeutic target. Moreover, in this study PD98059 (an ERK kinase (MEK)/ERK inhibitor) was used as a tool to further evaluate and characterize the effect of blockade of ERK activation on the proliferation of cutaneous SCC cell lines (DJM-1).

Chapter 2: Materials and methods

2.1 Tissues samples

Surgically resected specimens used for this study included 5 normal human skins obtained from healthy patients undergoing plastic surgery, 10 each of well- differentiated (Borders 'grade 1-2) and poorly-differentiated (Borders 'grade 3-4) SCC. All tissue specimens were selected from the files of the Department of Dermatology & Plastic and Reconstructive Surgery, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University. Informed consent and institutional review board agreement were obtained. For each formalin fixed and paraffin embedded tissue blocks, several 4-μm sections were cut. One section was stained with H&E for histological examination, and the others were used for immunohistochemical staining.

2.2 Antibodies and reagents

The Phospho-p44/42 MAP Kinase (Thr202/Tyr204) rabbit polyclonal antibody was purchased from Cell Signaling Technology (Beverly, MA,

USA). PD98059 (a MEK/ERK inhibitor) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary antibody and conjugate were included in VECTASTAIN Elite universal ABC kit PK-6200 (Vector Laboratories, Burlingame, CA, USA).

2.3 Immunohistochemical studies

Immunohistochemical staining was performed using the standard streptavidin-biotin-peroxidase complex method. Briefly, formalin-fixed 4 μm-thick paraffin sections were deparaffinized and subjected to antigen retrieval by microwaving in 10 mM of citrate buffer (sodium citrate, pH 6.0) for 15 min. The sections were then treated with 0.3% hydrogen peroxide in methanol for 20 min at room temperature to block endogenous peroxidase. After washing in phosphate-buffered saline (PBS), unspecific binding sites were blocked with 5% normal horse serum at room temperature for 1 hr. Excess serum was deleted from the sections. The tissues were then incubated with the primary antibody at 1:100 dilutions at 4°C overnight. Following washing with PBS, the sections were incubated with biotinylated horse-anti rabbit IgG at a dilution of 1:200 for 30 min at

room temperature. The slides were rinsed and incubated with the avidin/biotin complex at room temperature for 60 min. Visualization of the peroxidase reaction was achieved with diaminobenzidine (DAB), followed by counterstaining with Giemsa.

A negative control slide for each tissue was incubated with non-immunized horse serum to replace the primary antibody.

2.4 Evaluation of Immunohistochemical Staining

Only nuclear staining was considered positive for p-ERK1/2. A percentage score was measured by determining the percentage of positive tumour cells in >1000 tumour cells in >6 fields. The extent of immunoreactivity was evaluated in a semiquantitative manner using the following scale: Grade 1: < 5% of cells p-ERK1/2 positive; Grade 2: 5–25% of cells p-ERK positive; Grade 3: 26–50% of cells p-ERK1/2 positive; Grade 4: >50% of cells p-ERK1/2 positive. Sections were examined in a double blind manner to reduce bias and ensure consistency of examination.

2.5 Cell line and culture conditions

The human cutaneous squamous carcinoma cell line DJM-1 [46] was used in this study. The cells were routinely cultured in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS; Sigma, Deisenhofen, Germany) and antibiotics (penicillin, 100 U/ml and streptomycin, 100 μg/ml) at 37°C in a 5% CO_2 incubator. Cells were trypsinized and subcultured when they were approaching confluency.

2.6 Effect of PD98059 on cell proliferation

Confluent cells were harvested with an EDTA trypsin solution, and re-suspended to appropriate concentrations in MEM medium containing 10% fetal bovine serum. After Seeding $1x10^4$ cells/1ml growth medium in each well of a 24-well culture plate, cells were incubated 24h to allow for attachment. Prior to addition of inhibitors, cells were cultured in serum-free MEM for 24hr to induce a quiescent state. Cells were then incubated for 24, 48 and 72 hr in serum-free MEM containing either 5 μM, 10 μM, 20 μM, or 30 μM PD98059 (dissolved in dimethylsulfoxide

(DMSO); final concentration in medium were < 0.1%). In addition, Cells incubated with serum-free MEM with 0.1% DMSO served as a control in this study. All experiments were performed in triplicate. The attached cell numbers were determined using a Coulter counter (Beckman Coulter, Fullerton, CA, USA).

2.7 Statistical analysis

Data are expressed as mean ± standard deviation. All experiments were performed in triplicate. Significant differences among the groups are determined using the Mann-Whitney U-test. A value of $P < 0.05$ was considered as significant.

Chapter 3: Results

3.1 Expression of p-ERK1/2 protein in normal control skin and normal skin adjacent to tumor

In the control specimens of normal skin, no positive staining was seen in the epidermis and hair follicle, p-ERK1/2 immunoreactivity was observed in luminal surface of the acrosyringium, and in luminal surface and the nuclei of luminal cell in intra-dermal portions of the eccrine sweat ducts. Weak nuclear staining was occasionally found in some vascular endothelial cells (Fig. 5). A similar expression pattern was obviously strengthened in normal skin adjacent to tumor (Fig. 6).

3.2 Expression of p-ERK1/2 protein in cutaneous squamous cell carcinoma

The results are summarized in Table 2 and illustrated in Fig 7. Positive nuclear staining for activated ERK1/2 protein was detected in all investigated SCC specimens, there was an obvious difference in the expression levels between poorly differentiated SCCs and

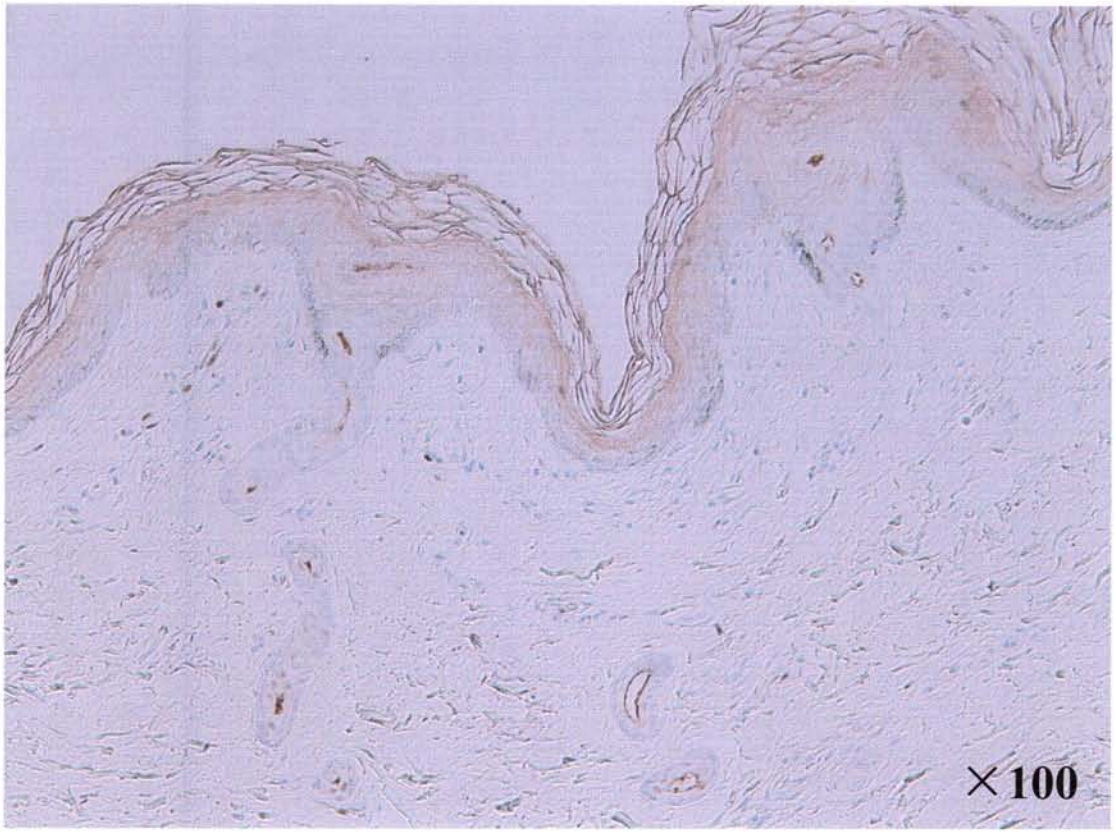

Fig. 5: Expression of p-ERK1/2 protein in normal control skin.

The expression was seen in luminal surface of the acrosyringium, and in luminal surface and the nuclei of luminal cell in intradermal portions of the eccrine sweat ducts. A few vascular endothelial cells with weak nuclear staining were also seen. No signal was seen in the epidermis.

well-differentiated SCCs. Immunohistochemical analysis showed that expression of activated ERK1/2 was significantly increased in poorly differentiated SCCs as compared with well-differentiated ones ($P < 0.05$).

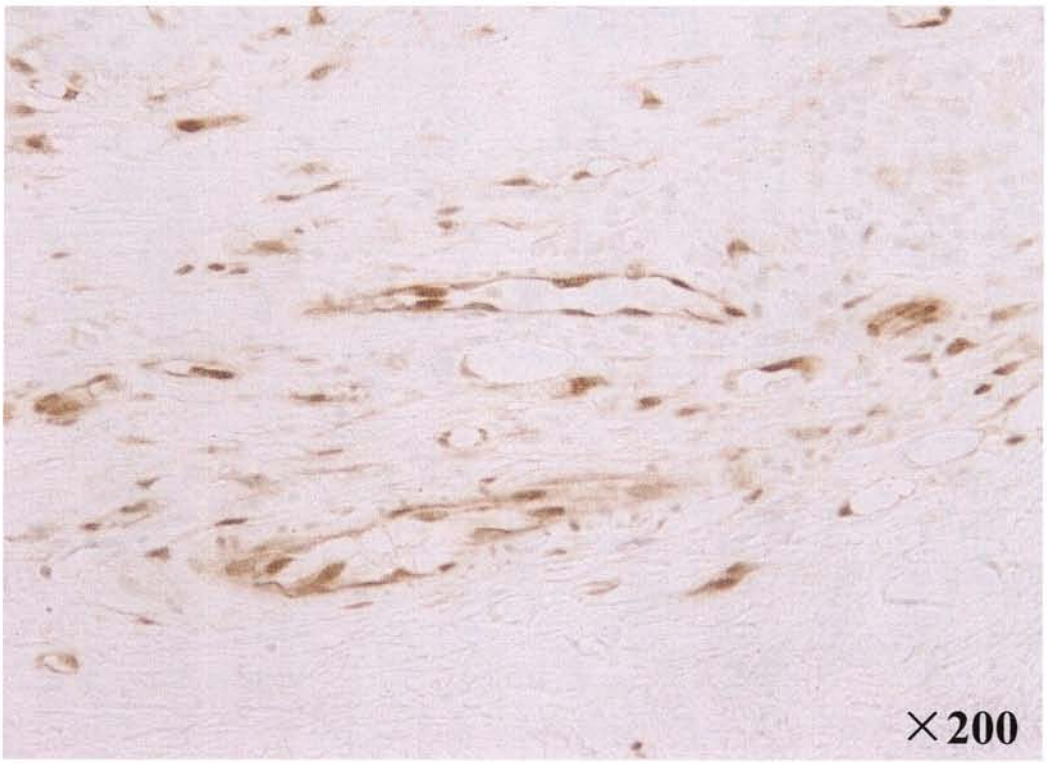

Fig. 6: Expression of p-ERK1/2 protein in vascular endothelial cells around tumor regions.

p-ERK1/2 nuclear staining was significantly increased in vascular endothelial cells adjacent to tumor in comparison to their in the normal control skin samples.

Table 2: summary of p-ERK expression in 20 cases of SCC

		p-ERK expression			
		Grade 1	Grade 2	Grade 3	Grade 4
Histological typing	total	(<5%)*	(5~25%)*	(26~50%)*	(>50%)*
Well-differentiated	10	2	6	2	
Poorly differentiated	10			3	7

P-ERK, phosphorylated extracellular signal-related kinase; SCC squamous cell carcinoma.*Percentage of p-ERK positive cells of tumor cells in the specimen.

3.3 Summary of p-ERK expression in 20 cases of SCC

As for the percentage of positive tumor cells, two cases were regarded as grade-1, six as grade-2 and two as grade-3 in well-differentiated SCCs, respectively. However, three cases were regarded as grade-3 and seven as grade-4 in poorly differentiated SCCs. Our findings demonstrated that the p-ERK expression was closely correlated with the degrees of tumor cell differentiation. Even in well-differentiated SCCs, only the peripheral cells of the tumor nests showed p-ERK1/2 positive, but the central keratin pearls showed negative immunoreaction.

3.4 Effects of PD98059 on cell proliferation

The results are shown in Fig 8. The human cutaneous squamous carcinoma cells, DJM-1 cells, were incubated with PD98059 for different periods of time at concentrations ranging 0~30 μM and the cell numbers were determined with a Coulter counter (Beckman-Coulter). PD98059 was shown to inhibit the proliferation of DJM-1 cells in a dose- and time-dependent manner. The results showed that SCC cells were extremely sensitive to growth inhibitory effects of PD98059, which at a

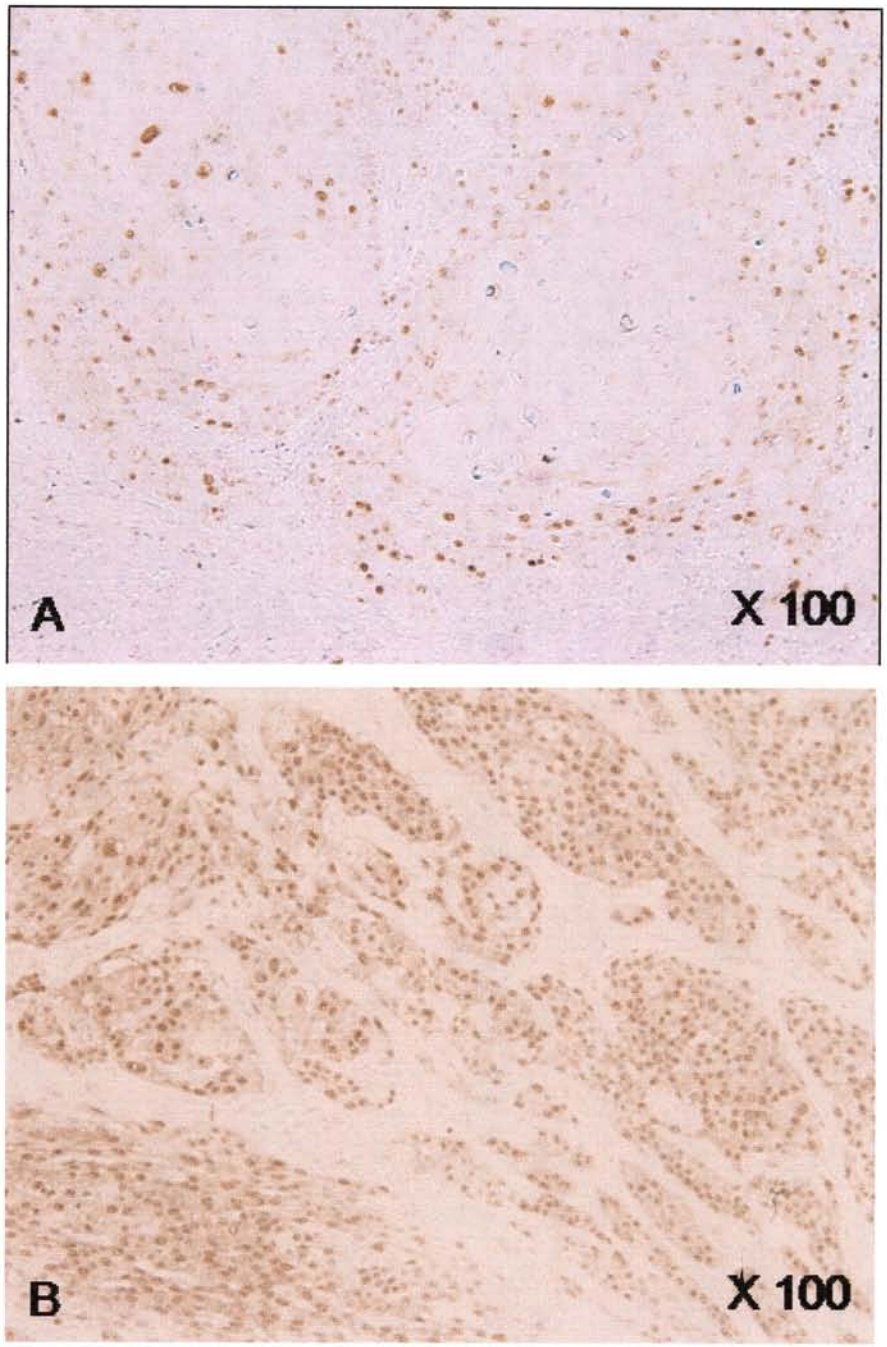

Fig. 7: Expression of p-ERK1/2 protein in cutaneous squamous cell carcinoma.

(A) In well-differentiated SCC, nuclear positive staining was noted in the less differentiated cells in the periphery of the tumor cell nests.

(B) In poorly- differentiated SCC, strong nuclear staining was present in the majority of tumor cells.

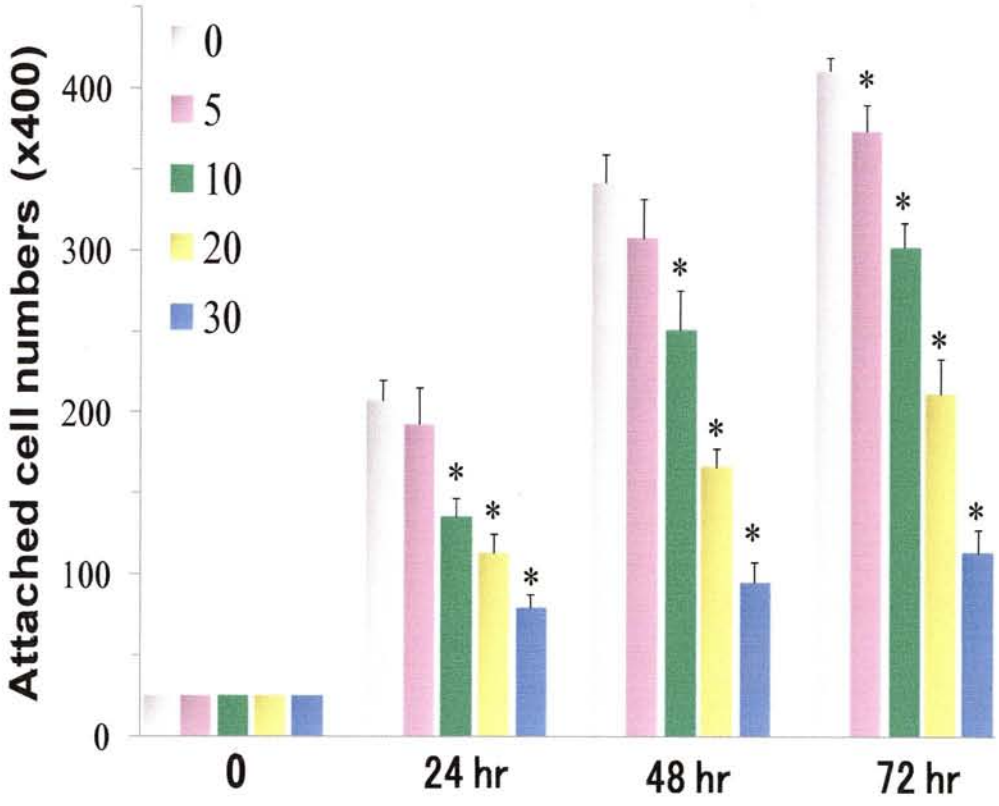

Fig. 8: Effects of PB98059 on DJM-1 cell proliferation.

Cells were plated in 24-well culture plates at a density of $1x10^4$ cells/well in serum-supplemented medium. After a 24 hr-attachment period, the cells were grown under serum-free conditions for 24 hr. Cells were then incubated in serum-free MEM with different concentrations of PD98059 (0, 5, 10, 20 and 30 μM). The numbers of attached cells were counted at 24, 48, and 72 hr after PD98059 treatments using a Coulter counter. Data are expressed as the mean ± standard deviation of three independent experiments, each of which was performed in triplicate. *$P < 0.05$ when compared with 0 (DMSO also).

concentration of 30 μM could almost completely suppress DJM-1 cells proliferation.

Chapter 4: Discussion

In the present study, nuclear staining of p-ERK1/2 protein was found in all SCC samples investigated. p-ERK1/2 expression was significantly higher in poorly-differentiated SCC than in well- differentiated SCC. Even in well-differentiated SCCs, the expression was also limited to the less-differentiated area of the tumor, and staining was rarely detected in large keratinized cells at the centre of cell nests or horny pearls. These results revealed that the expression levels of p-ERK1/2 were increased in accordance with decreasing grades of histological differentiation, suggesting that up-regulation of p-ERK1/2 expression reflect high degree of malignancy and proliferative activity of SCCs.

Immunohistochemical analysis showed that in the control specimens of normal skin, the expression of activated ERK1/2 was seen only at very low levels in some vascular endothelial cells, the epidermis and hair follicle did not show any positive reaction. In contrast, the expression of activated ERK1/2 was observed in all cases of SCC of epidermal origin examined in this study.

The data presented here suggest that ERK signaling pathway activation occurs during tumorigenesis and, possibly, plays a role in the oncogenic process.

Among MAPK pathways, The ERK pathway, known to be responsible for unregulated cell proliferation, is thus so far one of the best characterized and is closely related to human cancer. High levels of phosphorylated ERK1/2 have been reported in various types of human carcinoma cells [34~41]. The elevated expression of p-ERK1/2 observed in SCCs in this study is consistent with the results of previous studies indicating that increased expression of p-ERK1/2 was correlated with more aggressive tumor behavior and higher proliferative activity [37, 41, 44, 45, 47].

In order to further confirm the functional role of activated ERK1/2 in the proliferation in SCCs, we have performed experiments in vitro using MEK/ERK1/2 inhibitor PD98059 to treat DJM-1 in culture. We showed that DJM-1 could be almost completely suppressed by PD98059 at a concentration of 30 μM. These results corroborate previous experimental studies that suggest a critical role of p-ERK1/2 in the proliferation of

malignant tumors [42-45].

Multiple factors are associated the constitutive activation of the ERK1/2 pathway, including MEK-dependent and independent mechanisms [48, 49]. In cutaneous SCC cells, the MEK/ERK pathway inhibitor PD98059 completely inhibited cell proliferation, strongly suggesting that MEK-dependent mechanisms are involved. Based upon this data, we suggest the MEK/ERK pathway is important for cutaneous SCC cell proliferation.

Interestingly, in our studies a high level expression of active ERK1/2 was frequently found not only in tumor tissues, but also in vascular endothelial cells adjacent to tumor compared with their in the normal control skin samples.

The reason for increased activity ERK1/2 in vascular endothelial cells around tumor regions is not currently clear; a possibility is that many tumor cells produce growth factors such as transforming growth factor (TGF) - α and platelet-derived growth factor (PDGF) etc, which then stimulate the proliferation of tumor cells themselves (in an autocrine fashion) and also of the surrounding normal cells (in a paracrine fashion).

Thus, the elevated ERK activity observed in some non-tumorous tissues could be the result of such a paracrine stimulation of normal cells [50].

Several recent studies have indicated that various growth factors stimulated the ERK pathways and induced a significant increase in vascular endothelial cell proliferation in culture conditions in an ERK-dependent manner and such proliferation effect was completely blocked by PD98059 without toxicity [51].

Angiogenesis is necessary for tumor growth, invasion and metastasis. In addition to providing nutrients and oxygen and removing catabolites, proliferating vascular endothelial cell found in and around tumors produce multiple growth factors that can promote tumor cell growth, invasion, and survival [52,53].

On the basis of these observations and studies, we hypothesize that blocking ERK signaling pathway could have both direct and indirect effects upon both a tumor and its vasculature, and provides an effective strategy for human cutaneous SCC therapy.

Target-based therapies are widely considered to be the future of cancer treatment and much attention has been focused on developing inhibitors

of the Raf-MEK-ERK-MAPK signaling pathway and its upstream activators. ERK is a downstream component of an evolutionarily conserved signaling module that is activated by the Raf serine/threonine kinases. Raf activates the MEK1/2 dual-specificity protein kinases, which then activate ERK1/2. The mutational activation of Raf in human cancers supports the important role of this pathway in human oncogenesis. Additionally, the Raf-MEK-ERK pathway is a key downstream effector of the Ras small GTPase, the most frequently mutated oncogene in human cancers. Finally, Ras is a key downstream effector of the epidermal growth factor receptor (EGFR), which is mutationally activated and/or overexpressed in a wide variety of human cancers. ERK activation also promotes upregulated expression of EGFR ligands, promoting an autocrine growth loop critical for tumor growth. Thus, the EGFR-Ras-Raf-MEK-ERK signaling network has been the subject of intense research and pharmaceutical scrutiny to identify novel target-based approaches for cancer treatment [54, 55].

Because of its multiple roles in the acquisition of a complex malignant phenotype, specific blockade of the ERK pathway is expected to result in

not only an anti-proliferative effect but also in anti-metastatic and anti-angiogenic effects in tumor cells.

Activation of the ERK pathway occurs in response to integrin-mediated cellular adhesion to the extracellular matrix, which plays a critical role in both tumor metastasis and angiogenesis [56, 57]. It was recently reported that active ERK is targeted to newly formed focal adhesions after integrin engagement of v-Src activation, providing support for a role for ERK in regulation of adhesion [58]. Transfection of constitutively active MEK, which resulted in increased expression of matrix metalloproteinases 2 and 9 as well as cathepsin L, resulted in macroscopic metastases [59]. It is therefore not surprising that MEK inhibition in colon tumor models resulted in decreased invasiveness as well as inhibition of cell motility [60]. It is also anticipated that inhibition of ERK signaling will negatively impact angiogenesis. Such an effect is likely based on our knowledge of sustained activation of ERK being required for angiogenesis [61]. ERK activation is probably also required for growth factor-induced secretion of angiogenic growth factors from tumor cells [62, 63].

Because of its importance in cancer, the ERK pathway has been a focus

for drug discovery for almost 15 years with Ras, Raf and MEK as the main target. Recently potent small molecule inhibitors targeting the components of the ERK pathway have been developed. Among them, BAY 43 9006 (Raf inhibitor), and PD184352, PD0325901 and ADZ 6244 (MEK1/2 inhibitors) have reached the clinical trial stage [64].

Many studies have shown that inhibition of this pathway via MEK may be a most important target for therapeutic intervention in cancer [65]. MEK1 and MEK2 are closely related dual-specificity kinases, capable of phosphorylating both serine/threonine and tyrosine residues of their substrates ERK1/2. They are the only known catalytic substrates of Raf kinases. The fact that ERK is the only known substrate of MEK. When coupled with the observation that ERK is commonly activated in both tumor cell lines and patient tumors, has fueled strong interest in developing pharmacological inhibitors of MEK as a means to block ERK activation [66].

Additionally, it is believed that MEK is not frequently mutated in human cancer. However, aberrant expression of MEK is observed in many different cancers due to the activation of the Raf/MEK/ERK pathway by

upstream kinases and growth factor receptors as well as other unknown mechanisms. Specific inhibitors to MEK have been developed. The successful development of MEK inhibitors may be due to the relatively few phosphorylation sites on MEK involved in activation/inactivation. An advantage of targeting the Raf/MEK/ERK cascade is that it can be targeted without knowledge of the precise genetic mutation, which results in its aberrant activation. This is important as the nature of the critical mutation(s), which leads to the malignant growth of at least 50% of acute myeloid leukemias (AML) and other cancers, is not currently known (67). An advantage of targeting MEK is that Raf/MEK/ERK pathway is a convergence point where a number of upstream signaling pathways can be blocked with the inhibition of a single kinase (MEK).

In contrast to BAY 43 9006 (like other Raf inhibitor, BAY 43 9006 is not specific for Raf, it also inhibits other kinases), small molecule inhibitors of MEK are highly specific protein kinase inhibitors. Although the first two MEK inhibitors, PD98059 and U0126, were highly specific [68], they lacked the pharmaceutical properties needed to be successful clinical candidates. Nonetheless, these compounds have been invaluable

academic research tools for dissecting the MEK-ERK pathway and have provided enormous insight into the importance of ERK-MAPK signaling in cancer [69].

The first MEK inhibitor to enter clinical trials was PD184352, a non-ATP-competitive, highly selective inhibitor of MEK [60]. Preclinical evaluation found that PD184352 inhibited the growth of human colon cancer cells and human melanoma cells in athymic nude mice [60, 70]. Subsequent phase I and II clinical trials reported the most common toxicities were mild skin rash, diarrhea and fatigue. During the phase I trial, a partial response was seen in one patient with pancreatic cancer and 25% of patients with a variety of tumors had stable disease for greater than 3 months. Tumor tissues from treated patients showed significant reduction in activated phosphorylated ERK, indicating that the target was inhibited. These encouraging results prompted a phase II study in patients with advanced breast cancer, colorectal cancer (CRC) and pancreatic cancer. Unfortunately, the results of this trial were negative and PD184352 was determined to have poor pharmacokinetic properties [71]. However, when considered together with the significant body of positive

preclinical data as well as early indications from the phase I trial, it is still believed that MEK is a valid therapeutic target for the treatment of cancer. Thus, two second generation MEK1/2-specific inhibitors (PD325901 and ADZ6244) believed to have superior pharmacological and biopharmaceutical properties have been developed and are currently in clinical trials [55].

In contrast to the majority of protein kinase inhibitors, MEK inhibitors are non-ATP competitive inhibitors, which may account for their highly selective properties. Structural studies with an analog of 184352 in complex with MEK1 or MEK2 showed inhibitor binding did not perturb ATP binding, and instead, bound to a unique inhibitor binding pocket adjacent to the ATP-binding site. Inhibitor binding locked MEK in a catalytically inactive conformation. This recognition of MEK sequences that are not shared with other protein kinases, and their association with an inactivate conformation, account for MEK inhibitor target selectively [72].

Since activation of ERK1/2 pathway is associated with high degree of malignancy and proliferative activity of SCCs, patients with cutaneous

SCC displaying high levels expression of p-ERK will be a good candidates for such treatment. Moreover, the detection of p-ERK1/2 immunoreactivity in excised tumor sample could provide a straightforward way to select patient for these treatments and to monitor drug response.

In summary, we have shown that increased p-ERK is expressed in human cutaneous SCC and is related to proliferation activity. Inhibition of MEK/ERK signal pathway with PD98059 almost completely abolished SCC cell proliferation in vitro. Taken together, these results indicate that the MEK/ERK signal pathway may be an important potential therapeutic target in cutaneous SCC.

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