Topical application of 5-fluorouracil on attic cholesteatoma results in downregulation of keratinocyte growth factor and reduction of proliferative activity

Tomomi Yamamoto-Fukuda, MD, PhD; Mariko Terakado; Yoshitaka Hishikawa, MD, PhD; Takehiko Koji, PhD; Haruo Takahashi, MD, PhD

Department of Otolaryngology-Head and Neck Surgery (TY-F, MT, HT), Department of Histology and Cell Biology, Unit of Basic Medical Science (YH, TK), Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

Running head: 5-FU downregulate the growth of cholesteatoma

Address for correspondence and reprints requests:

Tomomi Yamamoto-Fukuda, MD

Department of Otolaryngology-Head and Neck Surgery,

Nagasaki University Graduate School of Biomedical Sciences,

1-7-1 Sakamoto, Nagasaki 852-8501, Japan

Phone: +81-95-849-7350

Fax: +81-95-849-7351

E-mail: tomomiYF@net.nagasaki-u.ac.jp

This paper was presented at the 9th international symposium on recent advances in otitis media, St. Pete Beach, Florida, USA, June 3-7, 2007.

Abstract

To investigate the cell-biological effect of topically applied 5-fluorouracil (5-FU) on middle ear cholesteatoma.

Twelve attic cholesteatomas were treated with topical application of 5-FU cream, two to five times with an interval of 2 weeks (5-FU group). The control group comprised 65 cholesteatoma that were not treated with 5-FU. All lesions were later excised surgically and processed for immunohistochemical analyses of Ki-67, keratinocyte growth factor (KGF) and its receptor (KGFR).

5-FU significantly reduced the expression of KGF, did not change KGFR expression, and significantly reduced the Ki-67 labeling index, relative to the control group.

The effect of 5-FU on cholesteatoma seems to be mediated, at least in part, through downregulation of KGF in stromal cells and reduction of the proliferative activity of epithelial cells.

Key words: Cholesteatoma, keratinocyte growth factor (KGF), Ki-67, 5-fluorouracil (5FU), immunohistochemistry

Introduction

Middle ear cholesteatoma is a pathological condition associated with otitis media[1, 2], accompanying hearing loss and occasionally facial palsy[3], and recurrence after surgical treatment is very common[4]. Cholesteatoma is a tumor-like mass consisted of keratinizing squamous epithelium, usually occurring in the middle ear and mastoid. Our understanding of the molecular mechanism underlying the pathogenesis of cholesteatoma is limited, but an important pathological process in this entity is active proliferation of epithelial cells[5-7], which is thought to be stimulated by various growth factors[8-10]. Among the latter, we focused in our previous study on the keratinocyte growth factor (KGF), and we indicated the possible involvement of both KGF and its receptor (KGFR) in enhanced epithelial cell proliferative activity and recurrence of cholesteatoma[11]. KGF, a unique member of the fibroblast growth factor (FGF) family, is a mesenchymal cell-derived paracrine mediator of epithelial cell growth[12], and its receptor KGFR is activated by binding to KGF, triggering a signal transduction pathway, which in turn stimulates the proliferation of epithelial cells[13]. Therefore, it seems quite reasonable to assume that KGF and KGFR play an important role in the proliferative stage of cholesteatoma.

While the pathogenesis of cholesteatoma has been analyzed and influence of cytokine has been emphasized, unfortunately, there was no suitable therapy except surgery. On the other hand, in 1985, Smith[14] treated cholesteatoma with 5-fluorouracil (5-FU) as an agent to suppress its abnormal proliferation, and reported its clinical effect. Furthermore, Wring et al.[15] confirmed the efficacy of 5-FU in animal

experiments and Sala[16] and Iwanaga et al.[17] reconfirmed its clinical efficacy and safety. Takahashi et al.[18] also tried this treatment for cholesteatoma using commercially available 5-FU topical cream, and reported its usefulness. However, the mechanism of action of 5-FU on cholesteatoma remains poorly understood.

In the present study, we examined the effect of 5-FU on the expression of KGF, KGFR and epithelial cell proliferative activity (Ki-67) in attic cholesteatomas that contain the head of the malleus and the body of the incus by immunohistochemistry.

Material and methods

Cases and Cholesteatoma specimens

The study subjects consisted of 77 ears with attic cholesteatoma. The age of the patients ranged from 10 to 85 years (average, 50 years). They represented all cases that were treated surgically at the Department of Otolaryngology, Nagasaki University Hospital between February 2002 and May 2006 (Table 1). Before surgery, 12 patients were treated with 5-FU. After cleaning the debris within or on the surface of the cholesteatoma as much as possible, 2-3 mm^3 of 5% 5-FU topical cream (Kyowa, Roche) was applied to the outer surface of cholesteatoma, transmeatally under the microscope, two to three times with an interval of 2 weeks (5-FU group). The other 65 cholesteatoma cases did not receive 5-FU treatment and served as the control group. For the control group, 36 patients were males and 29 patients were females, while for the 5-FU group, 10 patients were males and 2 patients were females. The average age was 59 years for the control group and 42 for the 5-FU group (Table 1). In both groups, cholesteatoma tissue was removed during surgery, and a part of it was sampled mainly from around the attic for this study.

The study protocol was approved by the Human Ethics Review Committee of Nagasaki University School of Medicine and a signed consent form was obtained from each subject for 5-FU treatment and surgery.

Tissue preparation

Cholesteatoma specimens were obtained during surgery from all 77 patients and used for immunohistochemistry. Each of the 77 specimens was fixed overnight in 10% buffered formalin at room temperature (RT) and embedded in paraffin. Serial sections were cut to 4 μm-thickness, and then placed onto 3-aminopropyltriethoxysilane-coated glass slides and adjacent sections were stained with hematoxylin and eosin and processed for immunohistochemical analysis of KGF, KGFR and Ki-67.

Immunohistochemistry for KGF and KGFR

Enzyme immunohistochemistry was performed to examine the expression of KGF and KGFR in tissue sections. Polyclonal antibodies against KGF and KGFR were prepared by immunization of rabbits against synthetic peptides in cooperation with Nichirei Co. (Tokyo, Japan). Cholesteatoma paraffin sections were deparaffinized with toluene and rehydrated by serially graded ethanol solutions. For KGFR detection, the slides were pretreated with 0.2% TritonX-100 in phosphate buffered saline (PBS) for 15 min at RT. After inactivation of endogenous peroxidase activity with 0.3% H_2O_2 in methanol, the sections were preincubated with 500 μg/ml normal goat IgG and 1% bovine serum albumin in PBS for 1 h to block nonspecific reaction with the first antibody. Then, the sections were incubated overnight with the first antibody at 0.5 μg/ml anti-KGF antibody or 8,000-fold dilution of anti-KGFR antisera, washed three times with 0.075% Brij 35 in PBS, and reacted with horseradish peroxidase (HRP)-goat anti-rabbit IgG for 1 h. After the slides were washed with 0.075% Brij 35 in PBS, HRP sites were visualized with 3, 3'-diaminobenzidine-4HCl

(DAB), Ni, Co and H_2O_2. For negative control, normal rabbit IgG or normal rabbit serum was used instead of the first antibodies, respectively, in every experiment.

Identification of proliferating cells

The nuclear antigen associated with cell proliferation was immunohistochemically detected using anti-Ki-67 antibody. For this purpose, sections were first deparaffinized, then the slides were autoclaved in 10 mM citrate buffer (pH 6.0) for 15 min at 120°C and processed in a manner similar to that described above, except for the concentration of anti-Ki-67 antibody (1:100) and the use of HRP-goat anti-mouse IgG (1:100) as a second antibody. As a negative control, some sections were incubated with normal mouse IgG instead of anti-Ki-67 antibody.

Quantitative analysis

For quantitative analysis, more than 1,000 cells were counted in random fields at 400 × magnification, and the number of Ki-67-positive cells was expressed as a percentage of positive cells per total number of counted cells [Ki-67 labeling index (LI); mean ± SD]. The staining intensities of KGF and KGFR were graded as (+) positive or (-) negative, compared to the background staining with normal rabbit IgG.

Statistical analysis

The percentage of the cells expressing KGF and KGFR was compared between the 5-FU group and control group for statistical significance using the Fisher-Exact-test. The Ki-67 LI of cholesteatoma tissues of the 5-FU group and

control group were expressed as mean ± SD. Differences in Ki-67 LI between the 5-FU group and control group were examined for statistical significance using the unpaired Student's t-test. A *P* value less than 0.05 denoted the presence of a statistically significant difference. All analyses were performed with a statistical software package (Excel 2003; Microsoft Corporation).

Results

Immunohistochemical localization of KGF and KGFR

In cholesteatoma tissues of the control group, intense staining for KGF was detected in stromal cells (Fig. 1A), which were mostly fibroblasts and infiltrating lymphocytes. In addition, a few KGF-positive cells were found in the epithelium. KGFR was predominantly found in the cells of the spinous layer and also in some basal and granular cells. The staining was exclusively localized to the plasma membrane (Fig. 1B). In addition, when the sections were reacted with preimmune normal rabbit serum instead of the first antibody, no staining was found (data not shown). In 7 out of the 12 specimens of the 5-FU group, KGF was detected in a few stromal fibroblasts (data not shown), but in 5 out of 12 specimens, KGF was not detected in any stromal fibroblasts (Fig. 2A). KGFR was found in epithelial cells in 6 out of 12 specimens of the 5-FU group (data not shown), while the remaining six cases were negative for the receptor (Fig. 2B).

The percentage of KGF-positive cases among the control group (90.5%, 57/63 specimens) was significantly higher than that of 5-FU group (58.3%, 7/12, $p < 0.05$) (Fig. 3). On the other hand, 34 out of 56 (60.7%) specimens of the 5-FU group were positive for KGFR while 6 out of 12 (50.0%) specimens of the control group were scored as KGFR-positive (Fig. 4). There was no significant difference in the incidence of KGFR-positive specimen between the two groups ($p=0.53$).

Ki-67 LI in cholesteatoma tissues of the 5-FU group and control group

Immunohistochemical analysis for Ki-67 was performed to compare the proliferative activity of cholesteatoma tissues of the 5-FU group and control group. In specimens of both groups, Ki-67-positive cells were found in the suprabasal and upper layers (Fig. 1C, Fig. 2C). In the tissues of control group, many Ki-67-positive cells were found in the basal and spinous layers (Fig. 1C). On the other hand in the specimens of the 5-FU group, Ki-67-positive cells were found in the basal layer mainly (Fig. 2C). The Ki-67 LI of the 5-FU group (37.5±0.2%) was significantly lower than that of the control group (49.5 ± 0.2%, $t=2.00$, $p<0.05$) (Fig. 5).

Discussion

The major findings of the present study were that topical treatment of attic cholesteatoma with 5-FU cream induced: *1)* significant downregulation of KGF expression, *2)* no change in KGFR expression, and *3)* significant reduction of epithelial cell proliferative activity.

In our previous study, we demonstrated that KGF was abundantly located in the granulation tissue underneath the epithelium of the cholesteatoma, which is rich in KGFR, indicating that cell proliferation in cholesteatoma is facilitated by chronic inflammation underneath the cholesteatoma epithelium[11]. The present study showed that KGF expression and Ki-67 LI of the 5-FU group were significantly lower than those of the control group. What do these findings mean? It is possible that 5-FU is taken up directly by epithelial cells, where it induces apoptosis of these cells. Alternatively, 5-FU could reduce the expression of KGF in stromal cells through its proapoptotic activity, which in turn reduces the proliferative activity of epithelial cells. 5-FU could induce apoptosis of both epithelial cells and stromal cells and downregulate the proliferative activity of epithelial cells in cholesteatoma through the paracrine action of KGF. In all of the mechanisms, 5-FU seems to arrest the growth of cholesteatoma tissues. In fact, in the previous study Kanamaru et al.[19] detected that 5-FU downregulated the growth of tympanic membrane and 5-FU inhibited the proliferation of fibroblasts of middle ear in a dose-dependent manner in vitro. The antiproliferative effect of 5-FU arises from its metabolites acting as metabolic blockers that inhibit thymidylate synthetase, which converts ribonucleotides to

deoxyribonucleotides, thus inhibiting DNA synthesis.[20] 5-FU acts selectively on the growth phases corresponding to DNA and RNA synthesis, respectively, in the cell cycle, therefore the cells in the synthesis phase are affected.[20]

5-FU did not downregulate the expression of KGFR. We postulate that the expression of KGFR in epithelial cells might serve to induce migration of epithelial cells against 5-FU-induced apoptosis.

On the other hand, the antiapoptotic effect of KGF was previously reported in human keratinocytes[21], and the studies suggested that KGF can forestall the ability of normal human keratinocytes to both initiate terminal differentiation and undergo features of apoptosis *in vitro*.[21] In contrast, in the present study, it seemed that the proapoptotic effect of 5-FU was opposed by the expression of KGF in stromal cells. However, we reported previously that there was no difference in the number of epithelial apoptotic cells in cholesteatoma and normal skin, and postulated that the production of KGF by epithelial cells could enhance their proliferative activity in cholesteatoma rather than inhibit their apoptosis[11].

Iwanaga et al.[17] studied the side effects of 5-FU in animal experiments and concluded that 5-FU treatment should be only indicated for cholesteatoma without eardrum perforation or cochlear or semicircular canal fistula. In our study, we selected only attic cholesteatomas in patients confirmed to be free of perforation at the bottom of retraction-pockets, and none of the patients developed any 5-FU-related serious side effect.

In the present study, as a control group, we only use cholesteatoma cases which did not receive 5-FU treatment. However, it is better to compare with

immunostainings of KGF, KGFR and Ki-67of their retroauricular skins as a control in both 5-FU-treated and non-treated groups. More strictly, control sample would have been harvested from a distant place like the site of retroauricular incision.

In conclusion, we analyzed the effects of 5-FU on the pathology and immunohistochemistry of cholesteatoma and found that this agent downregulated the expression of KGF in stromal cells of cholesteatoma and reduced the proliferative activity of epithelial cells. We believe that 5-FU treatment is effective for attic cholesteatoma and early-stage recurrence cases. We recommend 5-FU treatment for early-stage cholesteatoma with follow-up examination including computed tomography.

References

1. Harker LA. Cholesteatoma an evidence study. In: McCabe BF, Sade J, and Abramson M, editors. Cholesteatoma First International Conference. Birmingham: Aesculapius Publ, 1997; 308-309
2. Tos M. Incidence, etiology and pathogenesis of cholesteatoma in children. Adv Otorhinolaryngol 1988; **40**:110-117
3. Bagger-Sjoback D, Phelps PD. Cholesteatoma with extension to the cochlea. Am J Otol 1985; **6**:338-343.
4. Edelstein DR, Parisier SC. Surgical techniques and recidivism in cholesteatoma. Otolaryngol Clin North Am 1989; **22**:1029-1040.
5. Kim HJ, Tinling SP, Chole RA. Expression patterns of cytokeratins in retraction pocket cholesteatomas. Laryngoscope 2001; **111**:1032-6.
6. Sculerati N, Bluestone CD. Pathogenesis of cholesteatoma. Otolaryngol Clin North Am 1989; **22**:859-68.
7. Sudhoff H, Bujia J, Fisseler-Eckhoff A, Holly A, Schulz-Flanke C, Hildmann H. Expression of a cell-cycle-associated nuclear antigen (MIB 1) in cholesteatoma and auditory meatal skin. Laryngoscope 1995; **105**:1227-31.
8. Kojima H, Shiwa M, Kamide Y, Moriyama H. Expression and localization of mRNA for epidermal growth factor and epidermal growth factor receptor in human cholesteatoma. Acta Otolaryngol 1994; **114**:423-9.
9. Schulz P, Bujia J, Holly A, Schilling V, Kastenbauer E. Possible autocrine growth stimulation of cholesteatoma epithelium by transforming growth factor alpha. Am J Otolaryngol 1993; **14**:82-7.

10. Schilling V, Negri B, Bujia J, Schulz P, Kastenbauer E. Possible role of interleukin 1 alpha and interleukin 1 beta in the pathogenesis of cholesteatoma of the middle ear. Am J Otol 1992; **13**:350-5.
11. Yamamoto-Fukuda T, Aoki D, Hishikawa Y, Takahashi H, Kobayashi T, Koji T. Possible involvement of keratinocyte growth factor and its receptor in enhanced epithelial-cell proliferation and acquired recurrence of middle-ear cholesteatoma. Lab Invest 2003 ; **83**:123-36.
12. Finch PW, Rubin JS, Miki T, Ron D, Aaronson SA. Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. Science 1989; **245**:752-5.
13. Koji T, Chedid M, Rubin JS, Slayden OD, Csaky KG, Aaronson SA, *et al*. Progesterone-dependent expression of keratinocyte growth factor mRNA in stromal cells of the primate endometrium: keratinocyte growth factor as a progestomedin. J Cell Biol 1994; **125**:393-401.
14. Smith MF. The topical use of 5-fluorouracil in the ear in the management of cholesteatoma and excessive mucous secretion. Laryngoscope. 1985; **95**:1202-3.
15. Wright CG, Bird LL, Meyerhoff WL. Effect of 5-fluorouracil in cholesteatoma development in an animal model. Am J Otolaryngol 1991; **12**: 133-8.
16. Sala DT. Topical applications of 5-fluorouracil in the medical treatment of cholesteatoma of the middle ear. Ear Nose Throat J 1994; **73**: 412-4.
17. Iwanaga T, Tanaka F, Tsukasaki N, Terakado M, Kaieda S, Takasaki K, *et al*. Does topical application of 5-fluorouracil ointment influence inner ear function? Otolaryngol Head Neck Surg 2006; **134**: 961-5.

18. Takahashi H, Funabiki K, Hasebe S, Fukuda-Yamamoto T, Kaieda S, Iwanaga T, *et al*. Clinical efficacy of 5-fluorouracil (5-FU) topical cream for treatment of cholesteatoma. Auris Nasus Larynx 2005; **32**:353-7.
19. Kanamaru S, Nakamura T, Yamashita M, Magrfov A, Omori K, Ito J. 5-Fluorouracil ointment for the treatment of otitis media with effusion Laryngoscope 2007;**117**:215-9.
20. Hartman KU, Heidelberger C. Studies on fluorinate pyrimidines. XIII. Inhibition of thymidylate synthetase. *J Biol Chem* 1961;**236**:3006–13.
21. Hines MD, Allen-Hoffman BL. Keratinocyte growth factor inhibits cross-linked envelope formation and nucreosomal fragmentation in cultured human keratinocyte. J Biol Chem 1996; **271**:6245-51.

Figure legends

Fig. 1. Immunohistochemical detection of KGF and KGFR and Ki-67 in serial paraffin sections of middle ear cholesteatoma of the control group. ***A)*** KGF-positive cells in the stroma. ***B)*** KGFR-positive cells in the basal, spinous and granular layers in the epithelium. ***C)*** Ki-67-positive cells in the basal and spinous layers. Arrows: positive cells. Asterisks: positive layers. Magnification, ×200.

Fig. 2. Immunohistochemical detection of KGF, KGFR and Ki-67 in paraffin sections of attic cholesteatoma of the 5-FU group. ***A)*** KGF-positive cells are detected in few stromal cells. ***B)*** KGFR-positive cells are scarcely found. ***C)*** Ki-67-positive cells are detected in the suprabasal and upper layers, especially in the basal layer. Arrows: positive cells. Magnification, ×200.

Fig. 3. Correlation between the effect of 5-FU and expression of KGF in cholesteatoma. *Solid bars:* number of KGF-positive specimens, *open bars:* number of KGF-negative specimens, *left panel:* 5-FU group, *right panel:* control group. * $p < 0.05$.

Fig. 4. Correlation between the effect of 5-FU and expression of KGFR in cholesteatoma. *Solid bars:* number of KGFR-positive specimens, *open bars:* number of KGFR-negative specimens, *left panel:* 5-FU group, *right panel:* control group.

Fig. 5. Correlation between the effect of 5-FU and Ki-67 LI in cholesteatoma. Data are mean ± SD. *Left panel:* Ki-67 LI of the control group, *right panel:* Ki-67 LI of the 5-FU group. * $p < 0.05$.

Table 1. Cholesteatoma cases with/without 5-FU

	Number (Male/Female)	Age (yr)	Ki-67 LI	KGF positive cases (%)	KGFR positive cases (%)
Control group	65 (36/29)	59	49.5 ± 2.1	57 (90.5)	34 (60.7)
5-FU group	12 (10/2)	42	37.5 ± 2.2*	7 (58.3) *	6 (50.0)

* $p < 0.05$

Table 2. List of primary antibodies for immunohistochemistry

Antigen	Antibody	Working dilution
KGF	Polyclonal; Human KGF	0.5 μg/ml
KGFR	Polyclonal; Human KGFR	1:8000
Ki-67	MIB-1	1:100

Fig. 1

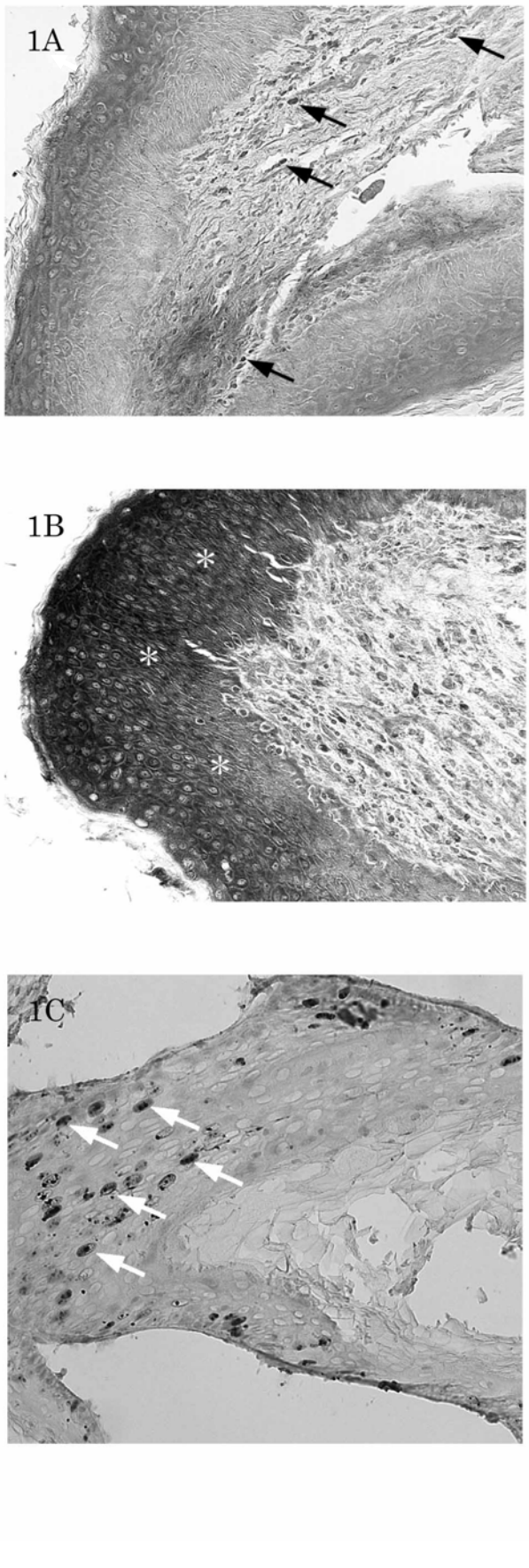
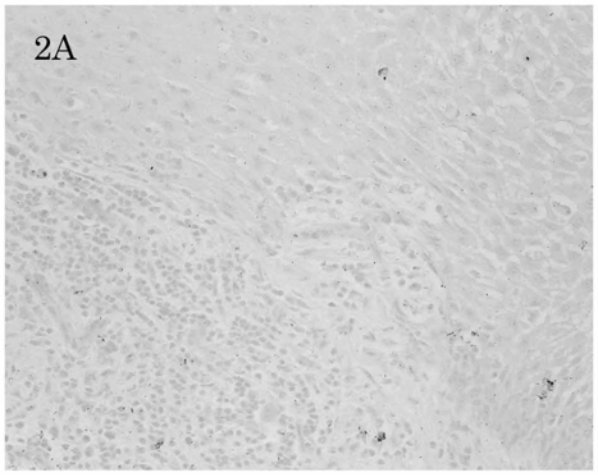
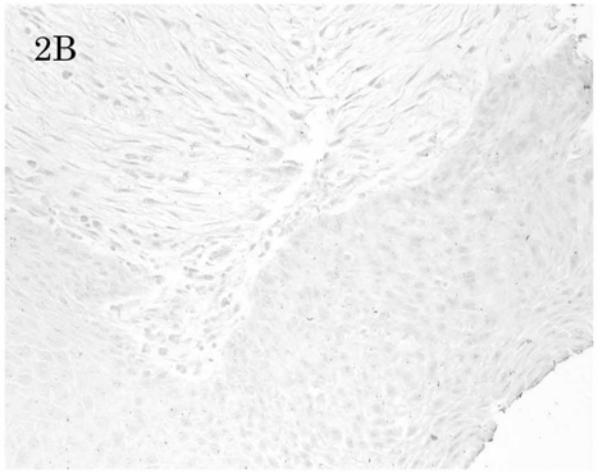


Fig.2





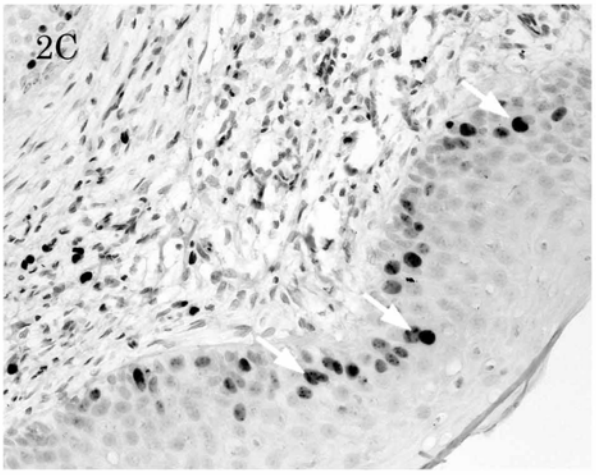


Fig.3

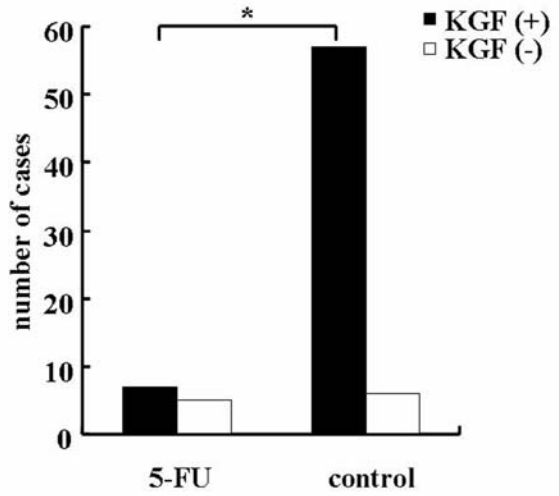

*
KGF (+)
KGF (-)
number of cases
60
50
40
30
20
10
0
5-FU
control

Fig.4

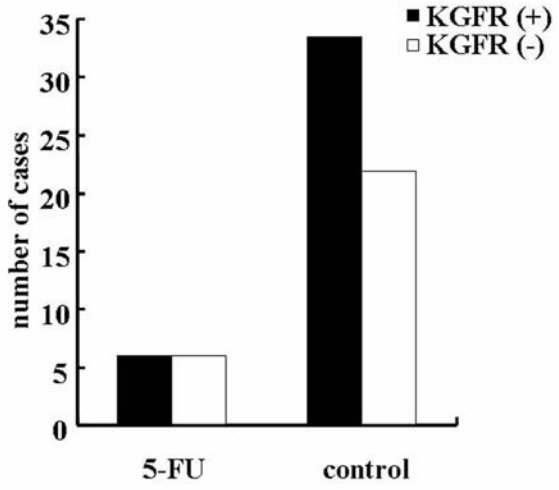

KGFR (+)
KGFR (-)
number of cases
35
30
25
20
15
10
5
0
5-FU
control

Fig.5