Establishment of 5-Fluorouracil Resistant OSCC Cell Lines: A Quantitative RT-PCR Analysis

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ABSTRACT

Objective: The purpose of the study is to establish 5-Fluorouracil (5-FU) resistant OSCC Cell Lines to evaluate the characteristics of these cells towards the targeted drug.
Methods: Fifty samples of oral squamous cells carcinoma have been taken from Hiroshima University Dental Hospital. The non-treated patients were included in the study. Patients, who were formerly treated were excluded. The study has been conducted by the establishment of cell line and cell culture, 5-FU resistant OSCC (Oral Squamous Cell Carcinoma) cell lines, drug sensitivity assay, protein extraction and western blotting, quantitative RT-PCR (Reverse Transcription Polymerase Chain Reaction) analysis, and statistical analysis. Student's t-test was applied to analyse the collected results.
Results: Correlation between 5-FU and protein levels of TS (thymidylate synthase) and DPD (dihydropyrimidine dehydrogenase) in the observed cell lines have showed insignificant results. Cell lines of HSC-3R, HSC-4R, Ca-9-22R and Ho-1-N-1R showed resistance of 2.7, 2, 11.2 and 2.6 folds, respectively through PCR technique.
Conclusion: OPRT (orotate phosphoribosyl transferase) and mRNA (Messenger RNA) have been reduced and resulted in low 5-FU. The study established 5-Fluorouracil (5-FU) resistant oral squamous cell carcinoma (OSCC) cell lines to evaluate the characteristics of these cells towards the targeted drug.

KEY WORDS: Resistance
Proteins
Resistant Cell Line
Cancer

Introduction

The factor of resistance in response to the chemotherapies and target treatments, particularly, in the cases of cancers, is emerging as a major concern of the oncology research and management. The performing mechanism of the resistance, developing towards the cellular operations, has become a major interest in the contemporary research. Nonetheless, several factors have been identified to be involved in the process that may include the modifications in the target of the drug, activation of the survival pathways by the tumour cells and inefficacy of the cell death mechanism [1].

Studies have identified that the tumours, which are responsive to chemotherapy, can acquire resistance during treatment by the cytotoxic agents. This is clinically characterized by a short period of remission and response failure to the subsequent therapy. Against many drugs, a mechanism acquiring resistance is still unknown that is presumed to be depending on various factors. It has been suggested that these elements may include the origin of tumour cells, degree of resistance expressed by the cancerous cells, and the methods utilized for the resistant cloning. Over the years, the need for understanding the phenomenon of resistance towards the drugs has become necessary for further advancements in the field [2,3].

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5-fluorouracil (5-FU) is a well-known anti-cancer drug that is frequently prescribed for the management of oral squamous cell carcinomas or OSCCs [4,5]. Apart from oral cancers; 5-FU is also used in lung, head, neck, mammary glands, and gastrointestinal cancers. The action mechanism of 5-FU is highly dependent on the enzymatic cascades. The enzymes that play significant part in the functioning of tumour suppressing role are thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD), orotate phosphoribosyl transferase (OPRT), and thymidine phosphorylase (TP) [6].

Many investigators have attempted to understand the mechanism in vitro and established various sub-lines from urine and human tumours, expressing resistance to drugs. The resistance has been observed as a response to repeated exposures of high concentration to target cells. 5-Fluorouracil (5-FU) has been used for treating oral squamous cell carcinoma (OSCC); whereas, the procurement of conflict is the major difficulty faced while treating OSCC. It is believed that the epithelial to mesenchymal transition (EMT) is associated with chemoresistance in different types of cancers. Therefore, the study has aimed to establish the 5-Fluorouracil resistant OSCC Cell Lines in order to evaluate the characteristics of these cells towards the targeted drug. However, the purpose is to enhance the literature, concerning vitro establishment of 5-FU resistant OSCC cell lines.

Materials and methods

Specimen

Fifty samples of oral squamous cells carcinoma have been obtained from Hiroshima University Dental Hospital because only 50 patients had been clinically followed up for last 3 years were preferentially chosen. These samples were obtained from the non-treated patients for the purpose of examination. The ethical approval is not required as only the non-treated patients were recruited. Only the specimens were collected, which were provided by Hiroshima University Hospital. The study has only analysed the samples of oral squamous cells carcinoma, but not analysed its impact on patients; therefore, no ethical approval is required. Therefore, the patients, who were already treated, were excluded at the time of diagnosis, which left the sample size with only fifty patients. The study has been conducted on the basis of the protocol stated as follows:

Establishment of cell line and cell culture

Japanese Cancer Research Resources Bank (JCRRB) provided tumour cell lines for the assessment. The cell lines included human HSC-2, HSC-3, HSC-4, CA9-22, HO-1-N-1 HO-1-U-1 from human neck and head, and HSY and KSA from salivary gland tumours. Whereas, three cell lines from human head and neck tumours were produced in the laboratory; including Igaki, KKp and KKm. These cells, except for KKp and KKm, were cultured in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) with 10% heat-inactivated fetal bovine serum (Whittaker, Walkersville, MD, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco/BRL, Grand Island, NY, USA). The cultures were then incubated at 37℃ under 5% CO2 in air. On the other hand, KKp and KKm were cultured in Keratinocyte Basal Medium (Whittaker, Walkersville, MD, USA), supplemented with Keratinocyte supplement (Sigma Aldrich, USA).

Establishment of 5-FU resistant OSCC cell lines

HSC-3 cells were treated with 5-FU in a continuous manner. The dose concentration has been increased in stepwise pattern in every 3 weeks, for up to 5 μg/ml. HSC-4 cells were treated accordingly for 24 hours. Ca-9-22 cells were also treated with 5-FU in a continuous manner, but had the dose increment up to 1 μg/ml, in every 3 weeks. HO-1-N-1 cells were treated in a similar pattern with increase of 10 μg/ml, in very 3 weeks.

Drug Sensitivity Assay

Cells (1×104/100μl) were seeded onto plates with 96 wells. After the period of 24 hours, these cells were treated with various concentrations of 5-FU. Following the culture of 3 days, 20 μl of MTT solution was added to each well. The concentrations of 5-FU ranged from 0.13 to 100 μ. The plates from the wells were then incubated at 37℃ for 4 hours. Dark blue formazan crystals were dissolved in 50 μl of dimethyle sulphoxide (DMSO). The absorbance in each well has been calculated at 570 nm wavelength, using a micro-plate reader (Model 450, Bio-Rad, Hercules, CA, USA). Three independent experiments were also carried out for different concentrations while IC_{50} was calculated by dose effect analysis.

Protein Extraction and Western blotting

Protein extraction has been performed in a standard manner as described in the study by Chung et al. (2000). The protein samples (100 μg) were dissolved in the sample buffer and were subjected to 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE), followed by electro-transfer onto nitrocellulose filters. Immuno-detection was performed using anti-TS and anti-DPD mouse monoclonal antibodies and anti-MRP2 goat polyclonal antibody along with the peroxidase conjugated second antibody. Immunocomplexes were visualized with Western blotting detection systems (Amarsham Biosciences, UK). Western blotting is helpful for providing accurate quantification of the proteins; therefore, the results of western blotting were quantified through radioactive labels. Anti-α-tubulin mouse monoclonal antibody was used as the internal control. Trans-Blot Turbo transfer apparatus was used to blot protein gels along with the PVDF Midi transfer packs (Bio-Rad). Membranes were sent immediately to the blocking buffer for fluorescent

western blotting, and it was then incubated for one hour at room temperature. The membranes were imaged after the uniform blotting buffer for the entire protein transfer with stain free application.

Quantitative RT-PCR analysis

To observe mRNA expression levels in 5-FU resistant cell lines, real time fluorescence detection method was used. The real-time fluorescence result was perceived by a laser detector of ABI Prism 7700 Sequence Detection System (Perkin-Elmer, Foster City, CA). PCR amplification was performed by utilizing 96 wells in an optical tray and caps, with a 25 µl of final reaction mixture. The mixture was based on 600 nM in each primer, 200 nM probe, 5 U AmpliTaq gold, 2µM each of dATP, dCTP and dGTP, 40 0µM dUTP, 5.5 mM MgCl2, 1 u AmpErase uracil Nglycosylase, 1 × TaqMan bufffer A containing a reference dye.

The expression of mRNA expression has been quantified with real-time PCR using specific TaqMan probes for the genes that encode enzymes involved in the 5-FU metabolic pathway. Gold activation steps included incubation at 50℃ carried on for 2 minutes and at 95℃ for 10 minutes. The thermal cycle consisted of 40 rounds of amplification at 95℃, for 15 seconds, and at 60℃, for 1 minute. The primer and probe sequences were as follows:

OPRT:

5'-TCCTGGGCAGATCTAGTAAATGC-3' (forward primer),

5'-TGCTCCTCAGCCATTCTAACC-3' (reverse primer), and

5'-CTCCTTATTGCGGAAATGAGCTCCACC-3'

(probe).

β-actin:

5'-TCACCCACACTGTGCCCATCTACGA-3' (forward primer),

5'-CAGCGGAACCGCTCATTGCCAATGG-3' (reverse primer), and

5'-ATGCCCTCCCCCATGCCATCCTGCGT-3' (probe).

Statistical analysis

Statistical Package of Social Sciences (SPSS) version 20.0 was used to examine the results. Statistical difference between mRNA expressions of original and acquired resistant cell lines was analysed by applying Student's t-test. R/P ratio was calculated by mean value of mRNA expression in resistant cells divided by that in the parental cells.

Results

Expression of TS and DPD in head and neck tumour cell lines

Specificity of the antibodies in head and neck tumour cell lines had been noted by Western blotting. R/P ratio has been determined by remaining amount of a non-renewable resource, expressed in time. The bands are known to correspond to the recombinant TS and DPD, with the molecular weight of 36kDa and 108Kda, respectively TS and DPD proteins have been noted be expressed in all the evaluated cell lines as seen in Table 1.

Table 1. TS and DPD expressions of head and neck tumor cell lines.

HSC-2	HSC-3	HSC-4	Ca-9-22	Ho-1-N-1	Ho-1-U-1	HSY	Igaki	KSA	KKp	KKm
1.0	1.65	1.75	1.42	1.7	1.78	0.62	0.95	1.4	1.37	1.9
1.0	1.39	1.66	1.08	1.14	1.92	0.88	0.92	2.03	1.23	1.35

Sensitivity to 5-FU of head and neck tumour cell lines

IC_{50} of 5-FU was calculated in the following cell lines i.e. 12µg/ml in HSC-2, 8.5µg/ml in HSC-3, 11.5µg/ml in HSC-4, 12µg/ml in HSY, 80µg/ml in Ho-1-N-1, 11µg/ml in Ho-1-U-1. Results have been depicted in the Figure 1.

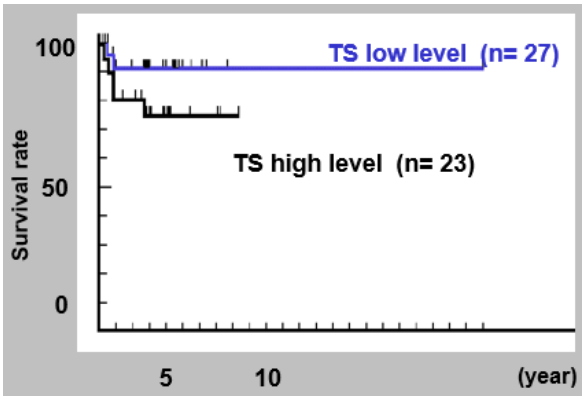
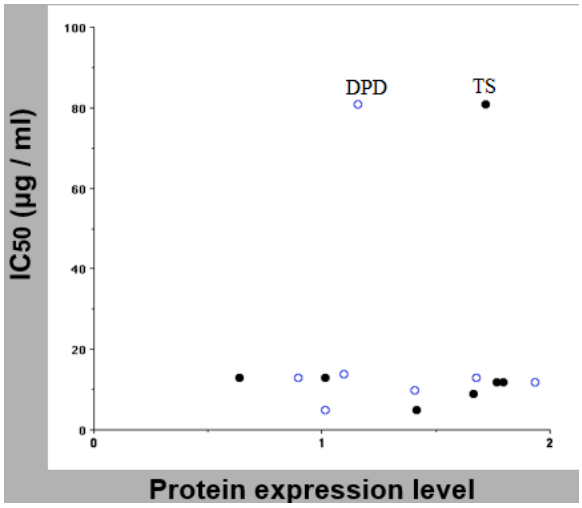


Figure 1. Growth inhibition head and neck tumor cell lines by 5-FU.

Response to 5-FU and TS and DPD expressions in tumour cell lines

Correlation between IC_{50} of 5-FU and protein levels of TS and DPD in the observed cell lines have been observed in the study. No significant correlation has been expressed in Figure 2.

Figure 2. Correlation between protein levels IC_{50} of 5-FU concerning TS and DPD of cultured head and neck tumor cell lines.



Establishment of 5-FU resistant OSCC cell lines

To obtain 5-FU resistant cells, HSC-3, HSC-4, Ca-9-22 and Ho-1-n-1 cells were treated with step-wise increasing concentrations of 5-FU. Obtained cell lines, HSC-3R, HSC-4R, Ca-9-22R and Ho-1-N-1R showed resistance of 2.7, 2, 11.2 and 2.6 folds, respectively.

Protein expression related to 5-FU resistance

To determine the relationship between increasing TS and DPD and 5-FU resistance, related proteins were semi quantified by Western blotting. It has been observed that TS expression was increased to 1.38 fold in HSC-4R as compared to its parental cells. However, the expression did not change in other cell lines. DPD protein had been noted to have a tendency to increase more than 1.12 fold in all cell lines. Results have depicted that these two proteins may relate to the 5-FU resistance in Table 2.

Table 2. Correlation between IC_{50} of 5-FU and protein levels of TS and DPD of cultured head and neck tumor cell lines

	HSC3 Reserves to Production ratio (R/P) ratio	HSC4 R/P ratio	Ca9-22 R/P ratio	Ho-1-N1 R/P ratio
TS	1.05	1.38	0.99	1.04
DPD	1.12	1.25	1.20	1.14

OPRT mRNA expression of 5-FU resistant cell lines

5-FU resistance can be defined as down-regulation of 5-FU anabolizing gene because of low degree of 5-FU incorporation in nucleic acid. OPRT mRNA expression was analysed by quantitative Real-Time PCR. It has been seen that OPRT expression of 5-FU resistant OSCC cell lines tends to be significantly lower than that of original cell lines among the three cell lines. There were no morphological and phenotype associated changes or alterations in proliferative activity. This suggests that low incorporation of 5-FU into nucleic acid has been due to the partial down-regulation of OPRT is displayed in Table 3. MRP2 protein is expressed at 190Kda. It has been observed that MRP2 protein increases to 2-fold in HSC-4 resistant cell line. It can be presumed that MRP2 might be one of the causes for resistance in HSC-4 cell line. Result has been displayed in Table 3. HSC-3, HSC-4, Ca-9-22 and Ho-1-N-1 were observed statistically insignificant as the p-values were greater than the level of significance (p-value>0.05).

Table 3. OPRT mRNA expression of 5-FU resistant OSCC cell lines and MRP2 protein expression of parental (P) and resistance (R) OSCC cell lines

OPRT	Expression of mRNA			
	Mean + SD	P - value	R/P ratio	MRP2 R/P Ratio
HSC-3 HSC-3R	1.0 0.73±0.26	0.28	0.51	0.76
HSC-4 HSC-4R	0.47±0.20 0.79±0.47	0.47	0.73	0.2
Ca-9-22 Ca-9-22R	2.24±2.41 1.49±1.21	0.72	1.69	0.69
Ho-1-N-1 Ho-1-N-1R	1.44±1.30 0.79±0.87	0.62	0.66	1.02

Discussion

5-FU is converted to fluorodeoxyuridine-5-monophosphate (FdUMP*) in vivo*. The converted product forms tight and stable complex with TS along with the 5, 10-methelene-tetrahydrofolate that ultimately inhibits the synthesis of DNA in tumour cells. Inhibition of DNA synthesis is mainly attributed to the methylation of deoxy-uridine-5-monophosphate (dUMP) into deoxy-thymidine-5- monophosphate (dTMP) that is performed by the action of TS [7,8]. TS plays an essential role in catalysing the reductive methylation of dUMP to dTMP, which are bound to provide the intracellular de novo sources for dTMP. In addition, they are responsible for initiating the most vital part of anti-cancer treatment that is the inhibition DNA biosynthesis in the cancer cells. Thus, the reaction catalysed by TS is considered highly contributing to the cellular proliferation and growth [9,10].

Studies conducted on bacteria, bacteriophage, yeast, viruses and vertebrates have expressed that TS is a dimer of identical subunits of 35Kda [11,12]. A study has examined the regulation of TS expression through a cell cycle directed events. It has been observed that the TS attain its maximal activity during the period of active synthesis of DNA. It asserts that expression of TS is directly associated to cell proliferation. Furthermore, it has elucidated the clinical significance of inhibition related with DNA synthesis [13,14].

TS is identified as chemotherapeutic target enzyme for malignant treatment; however, it is also an important prognostic index, correlating to the cancer activity [15,16]. Moreover, the degree of TS expression is also referred as it predicts the response to 5-FU therapy. It has been identified that low expression of TS corresponds to the greater response rate of 5-FU; whereas, high TS expression relates to the poor prognosis of gastric and colorectal cancers [17,18]. Likewise, the levels for TS have also been found to be associated with the survival in the cases of rectal and breast cancers [19,20]. A role of multidrug resistance-associated protein 2 (MRP2) has been identified in the manner [21].

One of the obstacles observed with the effective chemotherapy have been found to be depending on the development of drug resistance of tumours. In order to deal with these aspects, many studies have been performed to overcome the problem. The main approach for the identification of mechanism has involved the development of drug resistant cell line. 5-FU is one of the most frequently used chemotherapeutic drugs for OSCC [22,23]. It acts through its active metabolites that has been extensively discussed for its conversion mechanism and the driving enzymatic pathways [24,25]. As 5-FU exerts two action types on tumour cells, it is presumed that either one or both stimulates the mechanism and dominates the cytotoxicity depending on characteristics of pyrimidine metabolism [26,27].

A study established two sub-lines from human colorectal cancer, HCT-8 cells, by repeated exposure to 5-FU for 4 hours, or by continuous exposure for 7 days. One of the resistant sub-lines developed to 3-fold by the 4 hours exposure. They exhibited less incorporation of 5-FU to RNA, although the mechanism for this suppression has not been elucidated. The other sub-line developed by 7 days continuous exposure was developed to 7-fold more resistant. It further expressed less folypolyglutamate synthase, thereby causing lower levels of folypolyglutamates and expressing faster recovery of TS inhibition by 5-FU [27].

This study has involved multiple parameters for the 5-FU resistance. The cell lines that underwent the examination, such as HSC-3, HSC-4, Ca-9-22, and Ho-1-N-1, were treated for 3 weeks. They acquired the 2.7, 2, 11.2, and 2.6 folds of resistance, respectively. These resistances were weaker in comparison to those cell lines that have been developed in experiments by Chung et al. and Inaba et al. It has been observed in several studies that 5-FU related enzymes have an association with its resistance among the tumour cells, especially if the level of resistance is determined to be low.[28] Resistance to fluorouracil has been multifactorial. Studies have suggested that one reason can be attributed to the catabolism carried on in the target cell. It can be determined as the influencing factor for the responses of fluorouracil among the cancer patients [29,30].

In this study, the TS levels increased only in one cell line. It has complied with the findings of studies that reported the increase of TS expression as one of the factors in acquiring 5-FU resistance. It has been identified that all the cell lines expressing the DPD protein have showed an increase in the tendency to develop resistance. Therefore, in-

crease in the levels of DPD has been resumed to be responsible for more degradation of 5-FU in these cell lines [29]. This study has employed the novel quantitative real time PCR technique. It has provided with more accurate and reproducible quantification of gene expressions as compared to other conventional PCR methods. The expression of OPRT mRNA has been reduced in the resistant cell lines. It can be suggested that low incorporation of 5-FU to nucleic acid has been down regulated by anabolizing enzyme, OPRT. It is; however, unclear that in what manner the enzyme's subcellular localization and variable levels of its expression, at the cellular level, affects the reaction of 5 fluorouracil. The immunocytochemical analysis has indicated that the OPRT preferably localized into the complexes of Golgi bodies. It has been deemed that both the down regulation and over expression of the enzyme OPRT makes the cells vulnerable to be exposed to the 5-FU. However, no immediate effect has been observed where DNA may have been going through damage. It primarily indicates that expression of OPRT levels may have significant role in articulating the response to chemotherapy, based on 5-FU, at the cellular levels. The study has also investigated the MRP2 protein in the resistant cell lines. It has been indicated that HSC-4 cell lines show 2 – fold increase of MRP2 protein in comparison to the parental one. Thus, the action of MRP2 protein has been recognized to play an important role in the cancerous cell lines, by increasing efflux of 5-FU from the cells [30].

The study has established 5-FU resistant OSCC cell lines by step-wise increasing concentration of 5-FU for characterizing the mechanism of resistance in oral squamous cell carcinoma. It identified multiple parameters in 5-FU resistance. These cell lines included HSC-3, HSC-4, Ca-9-22, and Ho-1N-1, which showed resistance by 2.7, 2, 11.2, and 2.6 folds increase, respectively. However, these resistances were lower in comparison to others. Expression of OPRT and mRNA has reduced, resulting in low incorporation of 5-FU to nucleic acid causing down regulation of anabolizing enzyme; OPRT. MRP2 proteins have also increased to 2-fold in HSC-4 resistant cell line. The increase in expression of MRP2 can be suggested to be related to 5-FU resistance.

Conflict of Interest

I declare that we have no conflict of interest.

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