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A novel approach to rescue immune escape in oral squamous cell carcinoma: Combined use of interferon-γ and LY294002

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Abstract. Major histocompatibility complex (MHC) class I molecules have been found to be downmodulated in many tumors. The antigen-processing machinery (APM) genes, especially transporters associated with antigen processing (TAP)-1 and tapasin play important roles in the processing of class I antigens. In this study, we investigated the expression of TAP-1 and tapasin in oral squamous cell carcinoma (OSCC); the result indicated significant down-regulation in the expression of these genes. Interferon (IFN)-γ treatment was applied. After the addition of IFN-γ, unexpectedly, the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway was activated, which induced the proliferation of tumor cells. With the combined application of LY294002 (specific inhibitor of AKT signaling) and IFN-γ, tumor cell apoptosis was induced and the expression of TAP-1 and tapasin was still up-regulated. Hence, our method is a novel and efficient approach to use IFN-γ for rescuing the cells from immunosurveillance.

Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignant tumor of the oral and maxillofacial region. In spite of the recent advances in conventional treatment modalities including surgery, chemotherapy, and radiotherapy, the prognosis of OSCC is poor (1,2). Hence, finding an effective treatment method is a primary concern. Recent developments in the immunobased techniques have increased the potential application of $CD8^+$ cytotoxic T lymphocytes (CTLs)-based immunotherapy for OSCC. However, since abnormalities in the major histocompatibility complex (MHC) class I antigens are frequently found in human malignant cells, any step in the sequence of events that leads to the formation of a functional class I molecule can be targeted during the therapy (3-5). To our knowledge, defects in the expression and function of antigen-processing machinery (APM) genes may remarkably impair the processing of tumor-associated antigens (TAAs) and the presentation of TAA-derived peptides to $CD8^+$ T cells, thereby providing malignant cells with an immune escape mechanism (6,7). On the basis of this information, we hope to develop a treatment strategy for OSCC that would prevent the escape of tumor cells from immunosurveillance and improve the efficacy of T-cell-based immunotherapy by restoring the normal expression of APM genes (8). Among the components of APM, transporters associated with antigen processing-1 (TAP-1) and the chaperon tapasin play important roles. Deficiencies of TAP-1 and tapasin are frequently reported in different types of malignant tumors, which suggests that the down-regulation of TAP-1 and tapasin may be one of the most important mechanisms adopted by tumor cells to escape from immunosurveillance (9,10). However, it remains to be confirmed if this mechanism is operative in OSCC. Many groups have reported that the transcription of APM genes could be induced by cytokines such as, interferon (IFN)-γ (11), which is a type II IFN produced predominantly by T lymphocytes, natural killer T (NKT) cells, and natural killer (NK) cells (12). Accordingly, IFN-γ has been widely recognized as the most promising cytokine for inducing APM genes. Moreover, many reports have suggested that IFN-γ has an anti-proliferative effect on a wide variety of tumor cells (11). Thus, IFN-γ can be effectively used to induce immune response and provide protection against tumor development. However, till now the application of IFN-γ is limited to certain immune diseases and a small proportion of tumors like metastatic renal carcinomas. Although IFN-γ greatly increases the expression of APM components, the adverse effects associated with IFN-γ treatment remain to be clarified. In this study, we examined the expression change of APM genes in OSCC tissue samples and investigated the signaling pathway activated after IFN-γ treatment. Our future studies

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Table I. Correlations between Tap1 expression and the clinicopathological findings.

Classification	Case no.	Relative mRNA level	Non-parametric tests value	P-value
Gender				
Male	16	0.518±0.255	Z=-0.146	0.884
Female	14	0.498±0.193		
Smoking				
Yes	20	0.455±0.189	Z=-1.672	0.095
No	10	0.612±0.251		
Drinking				
Yes	22	0.499±0.219	Z=-0.188	0.851
No	8	0.528±0.235		
T stage				
T1	11	0.483±0.207	X^2=5.983	0.112
T2	9	0.492±0.212	d.f.=3	
T3	7	0.535±0.225		
T4	3	0.572±0.381		
N stage				
N0	12	0.439±0.175	X^2=0.044	0.978
N1	13	0.541±0.206	d.f.=2	
N2	5	0.581±0.339		
Clinical stage				
Stage I	4	0.417±0.809	X^2=2.434	0.487
Stage II	8	0.492±0.249	d.f.=3	
Stage III	15	0.478±0.190		
Stage IV	3	0.690±0.285		
Stage I+II	12	0.464±0.199	Z=-0.523	0.601
Stage III+IV	18	0.540±0.235		
Pathological differentiation grade				
Well	11	0.602±0.215	X^2=11.851	0.003[a]
Moderately	13	0.502±0.226	d.f.=2	
Poorly	6	0.342±0.117		

[a]Statistically significant difference.

will be aimed at identifying the possible ways of using IFN-γ effectively and efficiently for cancer therapy.

Materials and methods

Clinical tissue samples. From June 2009 to September 2009, we recruited 30 primary OSCC patients without prior radiotherapy or chemotherapy. The subjects were 18 men and 12 women aged between 35 and 82 years and with a mean age of 57.2 years. After providing informed consent, the patients underwent radical surgery at the department of oral and maxillofacial surgery of the Shanghai 9th Hospital. Surgical tissue samples, including cancerous tissue and adjacent non-malignant epithelium, were collected by previously described procedures. The adjacent non-malignant epithelia were collected from sites at least 2 cm away from the edge of tumor cells. The stage of disease was determined according to the tumor-node-metastasis (TNM) staging system of the International Union Against Cancer. The histological grade of the tumor was determined according to the degree of differentiation of the WHO histological criteria.

Cell lines and cell treatment. OSCC cell lines CAL27 cells were purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum

Correlations between tapasin expression and the clinicopathological findings.

Classification	Case no.	Relative mRNA level	Non-parametric tests value	P-value
Gender				
Male	16	0.452±0.180	Z=-1.228	0.219
Female	14	0.524±0.206		
Smoking				
Yes	20	0.467±0.194	Z=-0.815	0.415
No	10	0.538±0.199		
Drinking				
Yes	22	0.500±0.215	Z=-0.141	0.888
No	8	0.465±0.133		
T stage				
T1	11	0.436±0.174	X^2=2.891	0.409
T2	9	0.504±0.243	d.f.=3	
T3	7	0.560±0.201		
T4	3	0.487±0.080		
N stage				
N0	12	0.418±0.195	X^2=3.526	0.172
N1	13	0.523±0.177	d.f.=2	
N2	5	0.578±0.217		
Clinical stage				
Stage I	4	0.377±0.121	X^2=4.640	0.200
Stage II	8	0.464±0.295	d.f.=3	
Stage III	15	0.510±0.144		
Stage IV	3	0.613±0.119		
Stage I+II	12	0.435±0.247	Z=-1.950	0.053
Stage III+IV	18	0.527±0.147		
Pathological differentiation grade				
Well	11	0.649±0.173	X^2=17.949	0.000[a]
Moderately	13	0.467±0.110	d.f.=2	
Poorly	6	0.252±0.078		

[a]Statistically significant difference.

(FBS), 100 unit/ml penicillin and 100 μg/ml streptomycin. The cells were incubated under 5% CO_2 at 37˚C.

To detect the effect of IFN-γ, CAL27 cells were serum starved overnight. Then, IFN-γ was added at a concentration of 0, 10, 50, 100, 150 and 200 ng/ml. After 1 h, the cells were separated and an RNA extract was prepared.

To detect the effect of IFN-γ and a specific inhibitor for AKT signaling (LY294002) used jointly or separately, IFN-γ was used in a final concentration of 50 ng/ml as recommended by Setiadi *et al* (12) and LY294002 (10 μmol/l, as recommended in the manual provided by Beyotime Institute of Biotechnology).

Real-time PCR. Tumor tissue and adjacent normal mucosa samples were first ground in liquid nitrogen; subsequently, a total RNA extract was prepared for both the samples and TRIzol (Invitrogen) was added to this extract. For CAL27 cells, the first step was skipped and only TRIzol was used. Then, cDNA was synthesized from the RNAs by reverse transcription. cDNAs of TAP-1 and tapasin were amplified by PCR. The primers used for PCR were as follows: TAP-1, upstream primer 5'-GGACTTGCCTTGTTCCGAGAG-3' and downstream primer 5'-CGGATGCAGTAGCCTGGTGCTA TCG-3'; length of product, 75 bp. Tapasin, upstream primer 5'-AGTGTCCCTGATGCCAGCAA-3' and downstream

primer 5'-CGGAAACACAAGGCAGAGCAATTCCG-3'; length of product, 79 bp. ß-actin, upstream primer 5'-TCA CCCACACTGTGCCCATCTACGA-3' and downstream primer 5'-CAGCGGAACCGCTCATTGCCAATGG-3'; length of product, 295 bp. Real-time PCR was performed to detect the change in expression of the afore-mentioned genes.

Semi-quantitative Western blotting. After treatment with IFN-γ and LY294002 both jointly and separately, cells were washed twice with PBS and lysed in cold lysis buffer. Proteins (20 μg) were separated by 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). The membrane was blocked in a mixture of 0.1% Tris-buffered saline tween (TBST) and 5% skimmed milk for 1 h at room temperature. Then, the membrane was incubated overnight at 4˚C with mouse monoclonal antibodies to TAP-1 (ab10356; Abcam), mouse monoclonal antibody to tapasin (sc80647; Santa Cruz), rabbit monoclonal antibody to phospho-AKT (Thr308), AKT (pan), phospho-GSK-3ß (ser9), phospho-c-Raf (Ser259) (all antibodies were from Phospho-AKT pathway sampler kit no. 9916; CST), and with cyclin D1 (DCS6; CST) and ß-actin (all from Sigma) as internal references. Next, the membrane was treated with an horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h. Proteins were detected using enhanced chemiluminescence (ECL) detection system (Pierce Biotechnology) according to the manufacturer's instructions.

Cell proliferation and apoptosis assay. For cell proliferation assay, 50 ng/ml of IFN-γ was added to the medium of CAL27 cells for 0, 12 and 24 h. Then, cells were trypsinized and seeded at a density of $5x10^5$/well in a 6-well plate containing 10% FBS-DMEM. The cell count was determined at 24, 48, 72 and 96 h. The assay was repeated at least 3 times.

For cell apoptosis assay, both Western blotting and propidium iodide (PI)-Annexin-V methods were applied. After treatment with IFN-γ and LY294002, either individually or in combination, we prepared a protein extract of CAL27 cells. Western blotting was performed to detect cleaved-PARP, which is a specific marker for cell apoptosis. Meanwhile, the tumor cells were collected and doubled stained with PI and Annexin V to determine the ratio of apoptotic cells.

Statistical analysis. All data were analyzed by using the statistical software SPSS10.0 for Windows (SPSS Inc., Chicago, IL, USA). The correlation between relative mRNA expression and pathological characteristics was analyzed by non-parametric tests. Analysis of variance (ANOVA) method was applied to determine the significance of the cell number in different treatment groups. When P-value was <0.05, the difference was regarded as statistically significant.

Results

mRNA expression of TAP-1 and tapasin in OSCC tissue samples is low. mRNA level in different tissue samples was quantified by real-time PCR. The correlation between the relative mRNA expression and pathological characteristics of the OSCC tumor sample, such as TNM stage, clinical stage, pathological differentiation grade, and smoking and drinking was analyzed. Interestingly, positive correlation was found between the mRNA level and the pathological differentiation grade of OSCC (P=0.001) (Tables I and II). The relative mRNA expression of TAP-1 and tapasin in tumors of different pathological grades was analyzed. In comparison with the levels in the adjacent non-malignant epithelia, the TAP-1 and tapasin mRNA levels in the well-differentiated cancerous tissues were, respectively, 0.602±0.215-fold and 0.649±0.173-fold lower, those in moderately differentiated cancerous tissues were 0.502±0.226-fold and 0.467±0.110-fold lower, and those in poorly differentiated cancerous tissues were 0.342±0.117-fold and 0.252±0.078-fold lower. The difference in the expression level between different pathological grade groups was statistically significant (P<0.05) (Fig. 1).

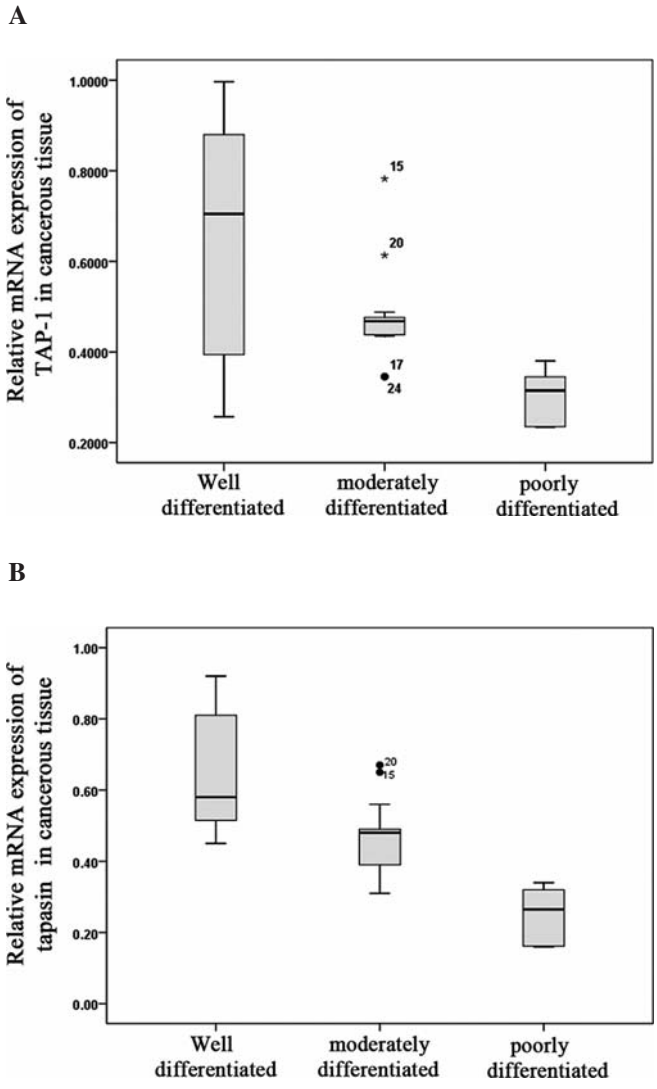

Figure 1. Relative mRNA expression of TAP-1 and tapasin in different pathological grades (A, B); the relative quantification of mRNA level for each sample was normalized against ß-actin. Boxes, distribution of expression values from the 25th to 75th percentile for each sample; horizontal lies in boxes, median values; whiskers, 5th and 95th percentiles; dots, outliers, P<0.05 in all comparisons.

IFN-γ exerts a dose- and time-dependent effect in increasing expression of both TAP-1 and tapasin. After a 1-h treatment

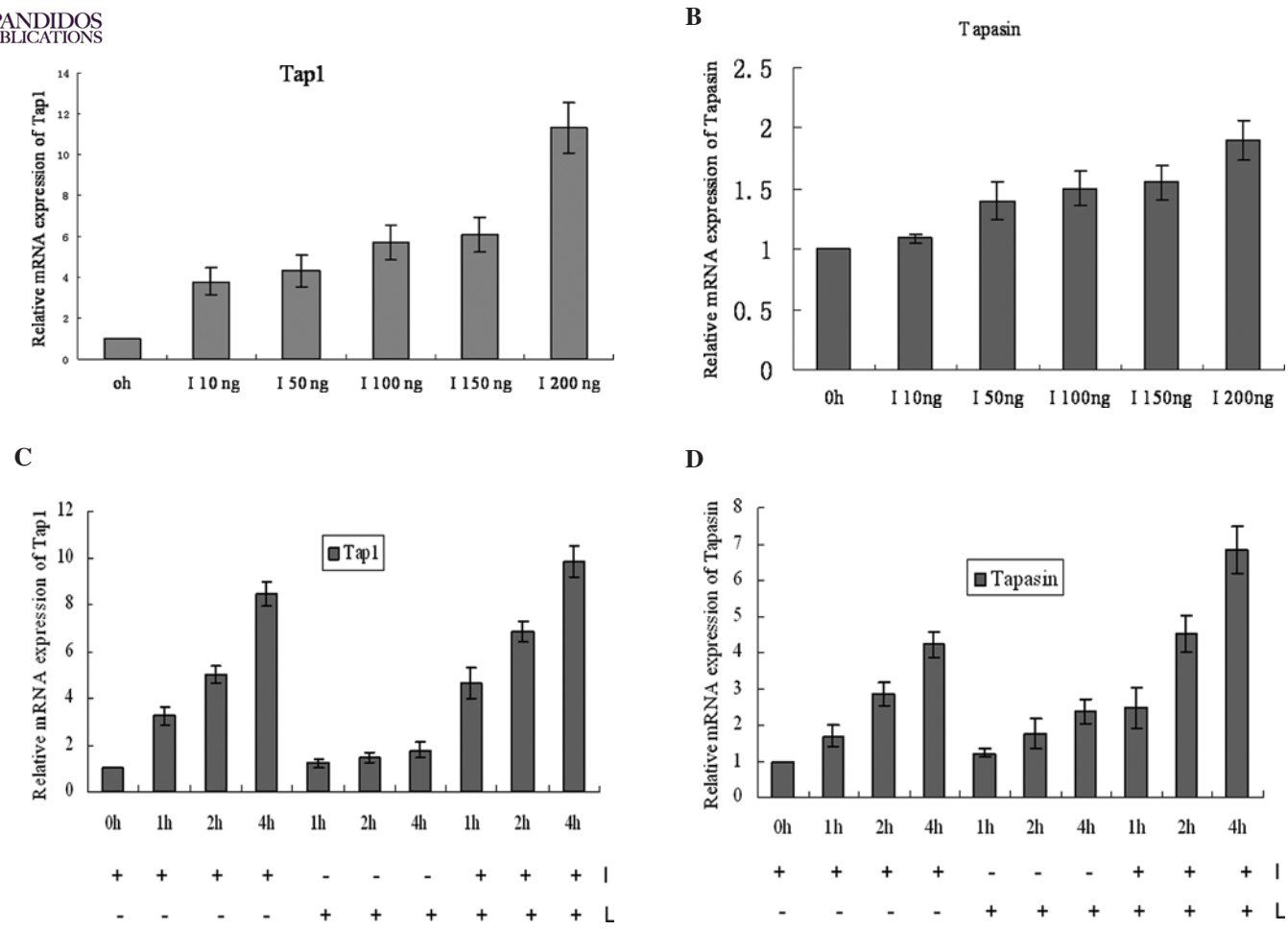

Figure 2. The mRNA expression of both TAP-1 and tapasin was enhanced after treatment with different concentrations of IFN-γ (I), and IFN-γ demonstrates a significant dose-dependency (A). A time-dependent effect was also observed after treatment with IFN-γ (I) and LY294002 (L) individually or together (B).

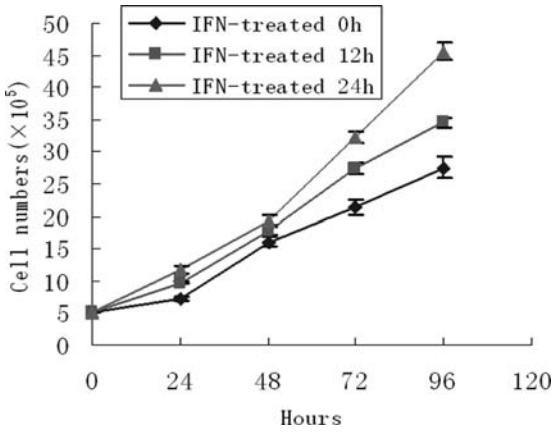

Figure 3. Cell growth curve of CAL27 cells after IFN-γ treatment for 0, 12 and 24 h. The results indicated an acceleration of CAL27 cell growth. At 72 and 96 h, the number of tumor cells was statistically different (P<0.05).

with IFN-γ in different doses, the relative mRNA expression of both TAP-1 and tapasin was analyzed. IFN-γ showed a significant dose-dependent effect (Fig. 2A and B). At the fixed concentration of 50 ng/ml, IFN-γ also showed a time-dependant effect in increasing the expression of TAP-1 and tapasin. Within 4 h, the relative mRNA expression of TAP-1 and tapasin reached 8.456±0.537-fold and 4.218±0.361-fold, respectively (Fig. 2C and D).

IFN-γ accelerates the proliferation of CAL27 cell lines. The growth curve obtained from the cell proliferation assay revealed the acceleration of cell growth in IFN-γ-treated groups as compared to the untreated control group. At 72 and 96 h, the number of tumor cells was statistically different (P<0.05) (Fig. 3) and this result was in contrast to some other reports, which help us to hypothesize whether any signaling promote tumor cell proliferation was activated.

PI3K/AKT signal pathway is proved to be activated after treatment with IFN-γ. Western blotting revealed the activation of phospho-c-Raf, significant phospho-AKT formation and increased expression of both phospho-GSK3ß and cyclin D1 in CAL27 cells treated with IFN-γ, thereby indicating the activation of PI3K/AKT signal pathway (Fig. 4).

The expression of TAP-1 and tapasin increased markedly after IFN-γ treatment; moreover, PI3K/AKT signal pathway was activated and tumor cell growth was significantly accelerated. Hence, we hypothesized whether we could select an inhibitor that can block the activation of PI3K/AKT signal while preserving the expression of APM genes.

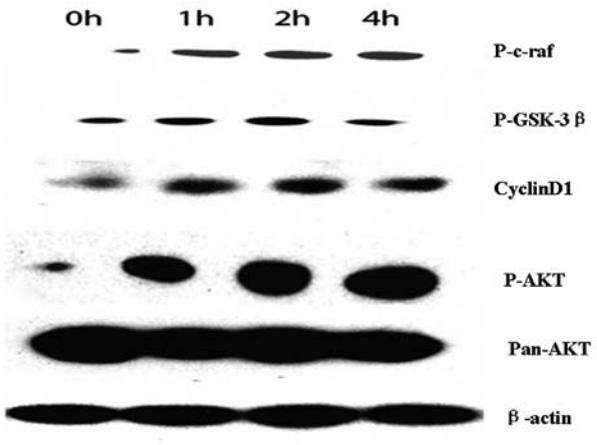

Figure 4. IFN-γ treatment resulted in a significant activation of PI3K/AKT signal pathway (which includes P-c-Raf, P-GSK-3ß, cyclin D1 and P-AKT).

LY294002 completely blocks the activation of the PI3K/AKT signal and exerts a synergic effect with IFN-γ in restoring the expression of TAP-1 and tapasin. Within an hour after the addition of LY294002, the PI3K/AKT pathway was blocked (Fig. 5A). Meanwhile, the result of PI-Annexin V staining revealed significant cell apoptosis as compared to that in the control. Cleaved-PARP was detected by Western blotting (Fig. 5B and C), thereby confirming the inactivation of PI3K/AKT signal.

Interestingly, 4 h after the addition of LY294002; real-time PCR revealed 1.784±0.324-fold and 2.375±0.312-fold increase in TAP-1 and tapasin expression, respectively. Therefore, we evaluated the combined effect of IFN-γ and LY294002; after 4 h, the expressions of TAP-1 and tapasin increased 9.861±2.318-fold and 6.831±1.872-fold, respectively, which were significantly different from that observed after individual treatments with IFN-γ and LY294002. Further, Western blotting revealed the same result (Fig. 5D).

Discussion

TAP-1 and tapasin serve important roles in the cell-surface expression of MHC class I molecules. Many groups have reported several tumors with significant deficiencies of TAP-1 and tapasin, suggesting that these proteins are involved in the

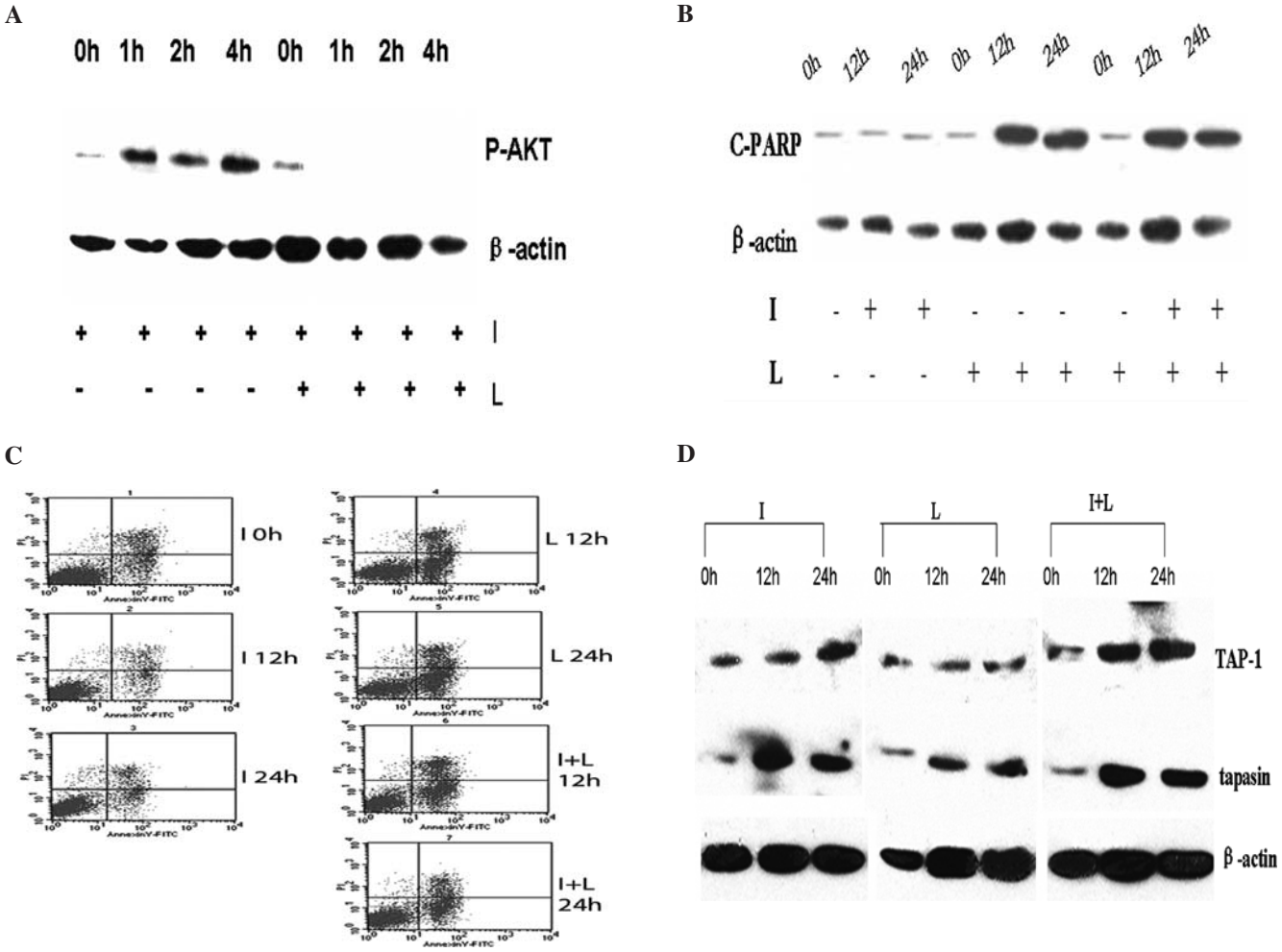

Figure 5. One hour after the addition of LY294002 (L), the PI3K/AKT pathway was blocked, no phospho-AKT appeared. (A) At 12 and 24 h after the addition of LY294002 in combination of IFN-γ or separately, Western blotting-detected appearance of cleaved-PARP (B), which confirmed IFN-γ$^+$ LY294002 induces tumor cell apoptosis instead of tumor cell proliferation, this result was also confirmed by PI-Annexin V staining (C). Western blotting revealed increased expression of TAP-1 and tapasin after treatment with LY294002 and IFN-γ individually or together (D).

 of MHC class I surface expression (10,13). Then, ıe status of these two genes in OSCC? In our study, both fresh tumors and their adjacent normal epithelial tissue samples were examined. A significant down-regulation of TAP-1 and tapasin was observed, and this result was in good agreement with the results of recent reports (14,15). Besides identifying the down-regulated gene, our group also reported a positive correlation between the expression of TAP-1, tapasin and the pathologic differentiation grades of OSCC. Low expression of TAP-1 and tapasin indicates a poor differentiation grade.

IFN-γ was once recognized to possess anti-tumor activity, which may be mediated by a direct cytotoxic effect on tumor cells or the activation of T lymphocytes and NK cells. However, new findings have indicated that IFN-γ could augment the immunogenicity of tumor cells by up-regulating the expression of MHC complexes, antigen processing machinery and tumor-associated antigens (12). It has been reported that IFN-γ-induced expression of TAP1 can enhance tumor-specific MHC class I-restricted CTL recognition of melanoma, small cell lung carcinoma, prostate carcinoma, and renal cell carcinoma (16,17). However, the molecular and clinical characteristics of the responses of OSCC to IFN-γ therapy are yet to be clarified. In our experiment, treatment with various concentrations of IFN-γ could restore the expression of TAP-1 and tapasin, and IFN-γ exhibited dose- and time-dependent effects in the *in vitro* model.

From the literature review, we know that IFN-γ can exert direct anti-proliferative and anti-metabolic effects on a wide variety of tumor cells. The molecular basis of these effects is the Stat1-dependent activation of gene encoding inhibitors that influence cellular progression (18,19). In contrast, our group found that IFN-γ promotes the proliferation of tumor cells in OSCC. Hence, we hypothesized that the PI3K/AKT signal pathway was activated. Our experimental results confirmed this hypothesis. After 1-h IFN-γ treatment, phospho-AKT, phosho-GSK3ß and cyclin D1 were formed and accumulated. PI3K/AKT signaling plays an important role in the survival and proliferation of tumor cells (20). It was a challenge to enhance the expression of APM genes while inhibiting tumor cell proliferation. In our study, we selected a small-molecule inhibitor LY294002, which was recognized as a specific inhibitor for AKT signaling. Western blotting and flow cytometry revealed that treatment of cells with LY294002 lead to tumor cell apoptosis. To our surprise, a synergistic effect for inducing expression of TAP-1 and tapasin was found, which makes it possible to gain the maximum advantage from IFN-γ. However, the specific molecular mechanism of this effect remains unclear.

In summary, we showed significant down-regulation of TAP-1 and tapasin in OSCC tissue samples. IFN-γ treatment restored the expression of these genes in OSCC cell line CAL27 and activated the PI3K/AKT signaling pathway. Combined treatment with LY294002 and IFN-γ not only preserved Tap1 and tapasin expression but also induced apoptosis of tumor cells. Our study provides a promising approach for future immunotherapy against OSCC.

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