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Original Article

DNA microarray reveals ZNF195 and SBF1 are potential biomarkers for gemcitabine sensitivity in head and neck squamous cell carcinoma cell lines

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Abstract: Gemcitabine is a potential chemotherapy drug for treatment of head and neck squamous cell carcinoma (HNSCC), however, the poor or partial response of HNSCC patients to gemcitabine demonstrated the urgent need for gemcitabine biomarkers to improve the therapy. In present work, 10 HNSCC cell lines were employed to figure out the biomarkers for gemcitabine sensitivity. The sensitivities of these 10 cell lines to gemcitabine and the basal expression of these cell lines was investigated, the correlation between gemcitabine response (IC50 dose) and gene expression was investigated by Pearson correlation and FDR estimation. The top seven positive genes responsible for gemcitabine sensitivity were validated by qPCR in these 10 HNSCC cell lines, while only two genes (SBF1 and ZNF195) were expression-correlated to gemcitabine response. Furthermore, ZNF195 expression was closely associated with gemcitabine sensitivity in the subsequent independent validation in cell lines from various types of cancer. Our work might provide potential biomarkers for gemcitabine sensitivity in HNSCC and various type of cancer.

Keywords: Head and neck squamous cell carcinoma, gemcitabine, DNA microarray, ZNF195, SBF1

Introduction

Head and neck squamous cell carcinoma (HNSCC) arises in the head or neck region (in the nasal cavity, sinuses, lips, mouth, salivary glands, throat, or larynx), and is the sixth leading cancer by incidence worldwide [1]. The most important risk factors for HNSCC are tobacco and alcohol consumption [2] and infection by high-risk types of human papillomavirus (HPV) [3, 4]. It is likely that approximately 650,000 cases will arise annually worldwide, and that only 40-50% of patients with HNSCC will survive for 5 years [5, 6]. Among these HNSCC patients, about two-thirds present with locally or regionally advanced stage disease, commonly involving regional lymph nodes. Induction chemotherapy or concurrent chemoradiotherapy (CRT) has advanced the treatment of locoregionally advanced (stage III/IV) HNSCC, allowing for functional organ preservation while maintaining or improving locoregional control (LRC) compared with radiotherapy (RT) alone [7-9]. Cisplatin, with good antitumor activity and the radiosensitizing property [10, 11], is regarded as a standard agent in combination with radiation or with other agents [6]. Gemcitabine, a drug with effective radiosensitizing roles against many cancer cells [12, 13], is one of the most widely used agents with proven efficacy in various types of cancer, including HNSCC [14, 15]. Therefore, gemcitabine is a potent drug for induction or concurrent CRT treatment of HNSCC patients. However, the poor or partial response to gemcitabine and the adverse effects of gemcitabine make it urgent to find biomarkers for gemcitabine sensitivity to improve the therapy and perform personalized therapy.

Previous studies have suggested that the expression of several DNA-repair related molecules, such as ribonucleotide reductase M1 (RRM1) [16], ribonucleotide reductase M2 [17], human equilibrative nucleoside transporter-1 (hENT1) and deoxycytidine kinase (dCK) [18] is correlated closely to gemcitabine sensitivity/resistance in non-small cell lung cancer (NSCLC)

or pancreatic ductal adenocarcinoma (PDAC). Moreover, inhibition of several kinases, such as PIM-3 [19], CK2 [20], sphingosine kinase-1 (SphK1) [21] and polo-like kinase 1 (Plk-1) [22], sensitizes PDAC cells to gemcitabine. In addition, gemcitabine sensitivity/resistance was proposed to be associated with the p53 mutation [23], NF-kB activity [24], the expression of multidrug resistant protein (ABCC5) [25, 26], apoptotic signaling molecules (Bcl2, BNIP3) [27, 28], sonic Hedgehog signaling-related molecules (ABCB2, MAP3K10) [29, 30], STAT3 [31], RON [32], NOTCH3 [33], and epithelial to mesenchymal transition (EMT)-related molecules (E-cad, Zeb-1) [34]. Although so many potential biomarkers for gemcitabine have been proposed, few were confirmed in prospective clinical trials. hENT1 expression was demonstrated not to correlate to gemcitabine outcome in phase 2 trial [35]. Additionally, there are few studies performed in HNSCC cells yet. Therefore, it is of great interest to figure out gemcitabine sensitivity-related biomarkers in HNSCC cells.

In present work, ten HNSCC cell lines with different sensitivities to gemcitabine were applied for DNA microarray analysis. The basal expression profiles were correlated to the gemcitabine sensitivities and those potential biomarkers for gemcitabine were proposed and independently validated in another 11 cancer cell lines. Our data strongly suggested that these gemcitabine sensitivity biomarkers could be expanded to other lineages and serve as very useful diagnosis markers in clinical therapy with gemcitabine.

Materials and methods

Cell culture

The human HNSCC cell lines, CEN-2, ECA-1, ECA-109, FADU, HEP2, HNE-1, HNE-2, HONE-1, KB, and TE-1, and another 11 cancer cell lines, HCT116, GP5D, RKO, COLO205, SK-Mel-25, LOVO, HCT15, GP2D, OVCAR-3, SK-Mel-28, BPH-1, and MG63 were used in this work. These cell lines were purchased from ATCC or China Center for Type Culture Collection. These cell lines were maintained in RPMI 1640 or DMEM medium (Gibco) supplemented with 10% FBS (Hyclone), penicillin (100 IU/ml) and Streptomycin (100 μg/ml) (Life Technologies) in a humidified atmosphere containing 5% CO_2 at 37°C. Cells in the exponential growth phase were used for all the experiments.

Determination of IC50 dose by MTS assay

Cells (1×10^3) were grown in 100 μl of RPMI 1640 or DMEM medium containing serum per well in a 96-well plate. After 24 h, the cells were treated with Gem (0, 1.0, 3.16, 10, 31.6, 100, 316, 1000, 3160, and 10000 nmol/L, respectively) for 144 h. Every treatment was triplicate in the same experiment. Then 20 μl of MTS (CellTiter 96 AQueous One Solution Reagent; Promega) was added to each well for 1 to 4 h at 37°C. After incubation, the absorbance was read at a wavelength of 490 nm according to the manufacturer's protocol. The cell viability was calculated relative to the untreated cells, respectively. The survival curves were plotted with the aid of GraphPad Prism 5.0 software via nonlinear regression. The IC50 calculation was performed with GraphPad Prism 5.0 software.

Microarray analysis

Cells (8×10^4) were grown in 2 ml of RPMI 1640 or DMEM medium containing serum per well in a 6-well plate. All the samples were homogenized with 1 ml Trizol (Invitrogen, Life Technologies) and total RNAs were extracted according to the manufacturer's instruction.

500 ng total RNA was used to synthesize double-strand cDNA and in vitro transcribed to cRNA, purified 10 μg cRNA was used to synthesize 2nd-cycle cDNA and then hydrolyzed by RNase H and purified. Above steps were performed with Ambion WT Expression Kit. 5.5 μg 2nd-cycle cDNA was fragmented and the single-stranded cDNA was labeled with GeneChip2 WT Terminal Labeling Kit and Controls Kit (Affymetrix, PN 702880). About 700 ng fragmented and labeled single-stranded cDNA were hybridized to an Affymetrix GeneChip Human Gene 1.0 ST array, which was washed and stained with GeneChip2 Hybridization, Wash and Stain kit (Affymetrix).

Data processing and significant genes prediction

Gene expression in multiple cell lines from individual or multiple lineages were measured. Expression of gene i across such cell line panel can be presented as, xi = {xi1, xi2, ..., xim}, where there are total m cell lines. Gene expres-

sion set, represented as a matrix X = {x1, x2, .., xn}, where n is the total number of genes and n >> m.

Cellular response to a drug (simply, drug response) are distinct in different cell lines even in same lineage. Therefore, to the same cell line panel, the drug response can be measured and quantitatively described as y = {y1, y2, ..., ym}.

Pearson correlation, denoted as R, was applied to measure the correlation/association between expression of ith gene across the cell panel xi and the drug response y.

Since n >> m, thus we can expect that there should be a number of genes can be identified as 'significantly' correlated with a given stochastically generated numbers yr and when giving yr for many times the number of called genes should be normally distributed around a positive integer k (± δ, the standard deviation). These genes called as significant here can be defined as the false positives. By random simulation, we are able to evaluate the distribution of numbers of the false positives. Therefore, with a given y from experimental measurement, when we found the number k' of positive genes is much larger than k, say k' > k + N × δ, N > 5, we are able to count the number of the true positives as k'-k. The false discovery rate (FDR) can be therefore produced, where we use the number of called genes when FDR = 50%, G_{FDR50}, to evaluate current learning and prediction.

To a given array *x* (1 × *N*), we can generate a stochastically distributed numeric matrix *Y* (*M* × *N*, and *M* >> *N*), where we can assert that a number of rows (*m*) can be found to considerably correlate to *x* only by chance (as the false positives).

Therefore, we expect that, in a given matrix *Y'* (which has been known to be not independent to *x*, for example, *x* and *Y'*, as different attributes, are both measured from same objects under a particular condition), *n* more rows (as the true positives) in *Y'* could be discovered to be correlated with *x*, since besides *m* rows are stochastically correlated to *x*, there are *n* rows indeed were affected by and associated with *x* with certain reasons. Here the false discovery rate (FDR) should be:

$$FDR = m/(m+n) \times 100\%.$$

To be more extensively accurate, we repeated generating the stochastically distributed numeric matrix Y_i many times and finally produced an averaged FDR curve along with the correlation coefficient (*r*), by which we are able to choose the best cutoff of r with considerably less false discoveries and more true positives.

Similarly, to a given matrix *Y'*, we are also able to calculate the FDR to a given array x by comparing with many randomly generated arrays x'_i (*i* = 1 to 500 in our case) with same distribution of *x*.

Notably, to a given pair of *x* and *Y'*, if the FDR were measured always around or over than 100%, we can make a conclusion that, to *x*, there are NO any rows in *Y'* truly correlated.

The gene expression was normalized by log2 transformed to the microarray data and the drug response was log10 transformed, the R value and FDR for each gene were calculated. Those genes with R ≥ 0.85 and FDR < 0.1% were denoted as true positive genes responsible for gemcitabine.

Quantitative real-time PCR (qPCR)

Total RNA above isolated was synthesized to cDNA using PrimeScript RT reagent kit with gDNA Eraser (Takara, RR074A) for RT-PCR with mixture of oligo-dT and Random Primer (9 mer). The primers used for qPCR validation were list in Supplementary Table 1. Real-time qPCR was performed on CFX-96 (Bio-lab), with endogenous control hActb. Gene expression was calculated relative to expression of hActb endogenous control and adjusted relative to expression in FADU cells or GP5D cells in independent validation.

Statistical analysis

R values were calculated using Pearson's correlation coefficient. The significant difference was calculated using Student's t-test.

Results

HNSCC cell lines display dramatic difference in gemcitabine sensitivities

Ten HNSCC cell lines were treated with 9 distinct doses of gemcitabine for 144 h. The cell viability was calculated relative to the untreated cells, respectively. The survival curves were

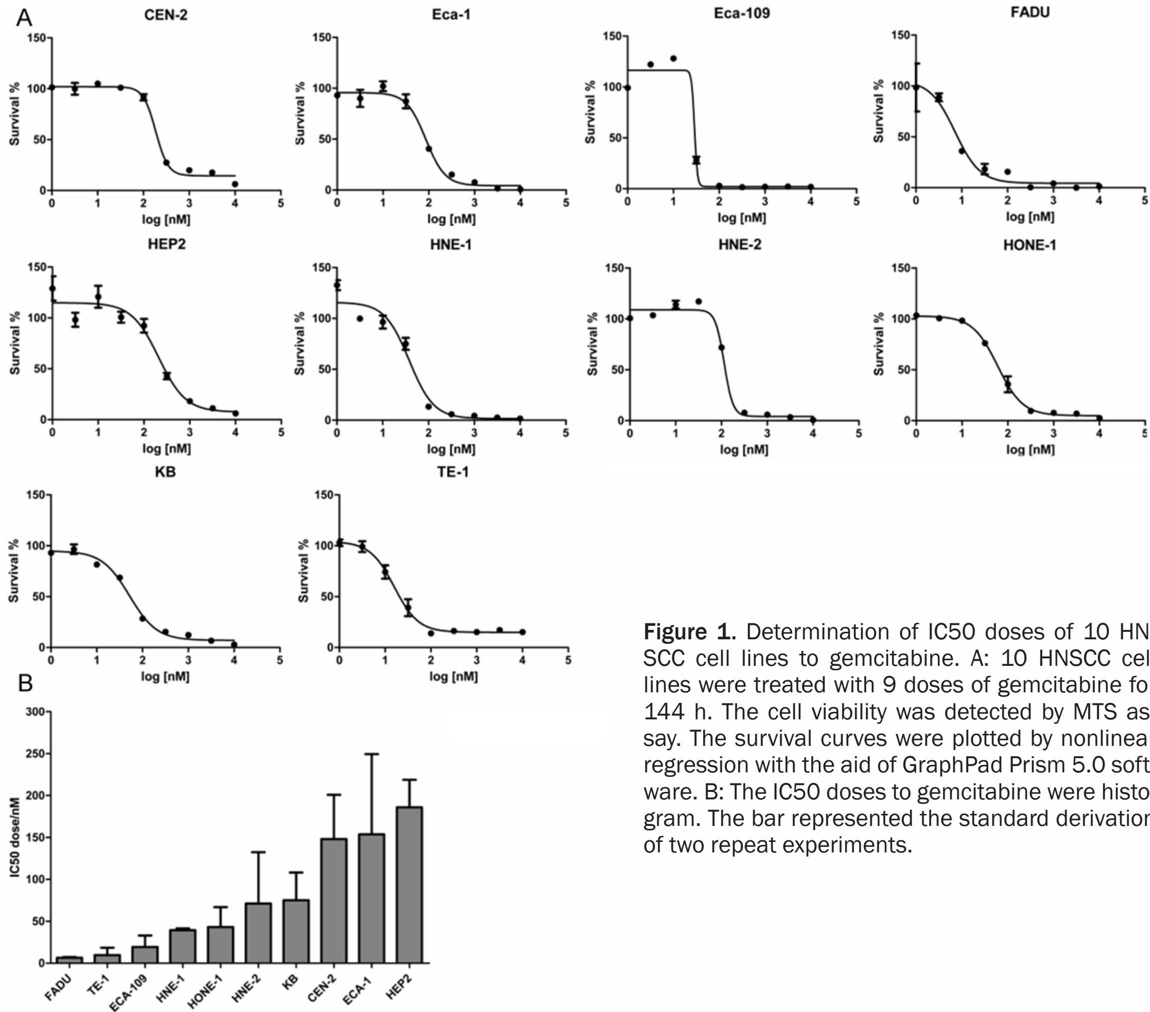

Figure 1. Determination of IC50 doses of 10 HNSCC cell lines to gemcitabine. A: 10 HNSCC cell lines were treated with 9 doses of gemcitabine for 144 h. The cell viability was detected by MTS assay. The survival curves were plotted by nonlinear regression with the aid of GraphPad Prism 5.0 software. B: The IC50 doses to gemcitabine were histogram. The bar represented the standard derivation of two repeat experiments.

Table 1. IC50 doses of 10 HNSCC cell lines to gemcitabine

Cell	R1[a]		R2[b]		MEAN	SD
	IC50 [nM]	R^2*	IC50 [nM]	R^2		
KB	51.75	0.9897	98.38	0.9623	75.1	33.0
ECA-109	28.96	0.9808	9.655	0.9831	19.3	13.7
HNE-1	37.95	0.9647	40.87	0.9965	39.4	2.1
HNE-2	114.5	0.9895	27.78	0.9925	71.1	61.3
FADU	7.122	0.9324	5.872	0.9945	6.5	0.9
HEP2	209.2	0.9483	162.8	0.9895	186.0	32.8
CEN-2	185.3	0.9888	110.7	0.9929	148.0	52.8
ECA-1	86.01	0.9808	221.3	0.9971	153.7	95.7
HONE-1	59.88	0.9941	26.36	0.9979	43.1	23.7
TE-1	15.84	0.9894	3.592	0.9652	9.7	8.7

[a]repeat 1; [b]repeat 2; *correlation coefficient.

plotted with the aid of GraphPad Prism 5.0 software via nonlinear regression (**Figure 1A**). The IC50 doses of these ten HNSCC cell lines for gemcitabine were histogram in **Figure 1B** and list in **Table 1**. The results showed that the sensitivities of these cell lines to gemcitabine were distributed in a nearly normal fashion, ie., FADU, TE-1 and ECA-109 cell lines were hypersensitive to gemcitabine, HNE-1, HONE-1, HNE-2 and KB cell lines were moderate sensitive to gemcitabine, while CEN-2, ECA-1 and HEP2 cell lines were resistant to gemcitabine.

DNA microarray analysis and prediction of significant gemcitabine-responsible genes

The basal expression of these ten HNSCC cell lines was investigated by DNA microarray. The prediction of significant gemcitabine-responsi-

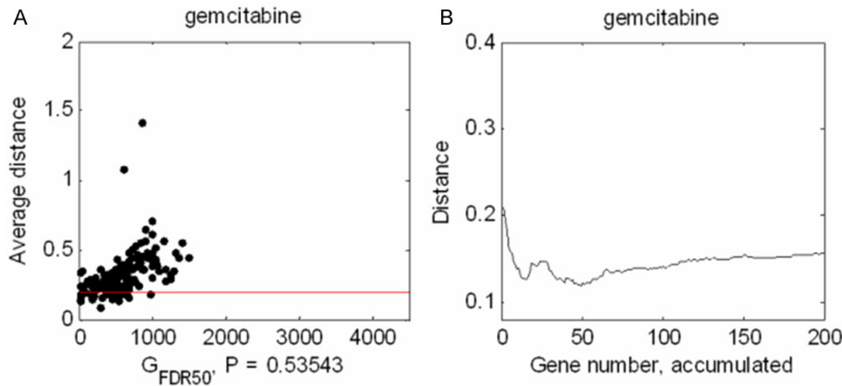

Figure 2. FDR estimation and minimal gene number for gemcitabine response prediction. A: G_{FDR50} prediction of gene for gemcitabine response prediction. There were 665 genes whose FDR value was below 50% in our calculation. B: Prediction of minimal gene number for gemcitabine response. The minimal gene number for gemcitabine response prediction was ten or so.

Table 2. The top 20 positive genes responsible for gemcitabine response

Gene	R*
P4HTM	0.9436
SBF1	0.9253
ZNF195	0.9251
FARSA	0.9232
GTF2IRD2B	0.9206
IL9R	0.8980
PIN4	0.9147
AC010492.2.1	0.9042
RP11-742N3.1.1	0.8987
RPL18AP3	0.8940
ARAF	0.9057
WASH7P	0.9052
MRPL54	0.9070
GPX1P1	0.9065
FAM156B	0.8704
RRM1	0.9019
EXOSC4	0.9026
MCM7	0.8980
CENPT	0.8497
MAP1LC3B2	0.8526

*R, Pearson correlation coefficient.

ble genes was performed, as described in Material and Methods. The gene expression was normalized by log2 transformed to the microarray data and the drug response was log10 transformed, the R value and FDR for each gene were calculated. The G_{FDR50} prediction and the gene number required for actual prediction of gemcitabine response in HSNCC cell lines were plotted in **Figure 2A** and **2B**, respectively. There were 665 genes whose FDR value was below 0.5, and at least ten genes were needed for perfect prediction for gemcitabine response in these HNSCC cell lines. Those genes with $R \geq 0.85$ and FDR $< 0.1\%$ were denoted as true positive genes responsible for gemcitabine. The top 20 significant gemcitabineresponsible genes were list in **Table 2**. In line with previous studies, RRM1 was predicted to be a gemcitabine-responsive gene in our results, which can be seemed as a positive control.

qPCR validation

Then the top seven genes (P4HTM, SBF1, ZNF195, FARSA, GTF2IRD2B, IL9R, PIN4) were validated in these ten HNSCC cell lines by qPCR. The R value between gene expression and gemcitabine response was calculated and the results showed that only two genes were significantly correlated to the gemcitabine sensitivity: ZNF195, R = 0.90, p = 0.0002; SBF1, R = 0.59, p = 0.037 (**Figure 3**). For the other genes, there was no significant difference ($p > 0.05$).

Independent validation in other cancer cell lines

Then these two genes potentially responsible for gemcitabine sensitivity were independently validated in 11 cancer cell lines from various types of cancer, including lung cancer, colorectal cancer, ovarian cancer, prostate cancer, melanoma and osteosarcoma. The expression of these two genes in these 11 cancer lines were investigated by qPCR assay (**Table 3**). The IC50 doses of these cancer lines were examined by MTS assay (**Table 3**). The correlation between gene expression and gemcitabine sensitivity was calculated by Pearson correlation with the aid of GraphPad Prism 5.0 and plotted (**Figure 4**). The results showed that

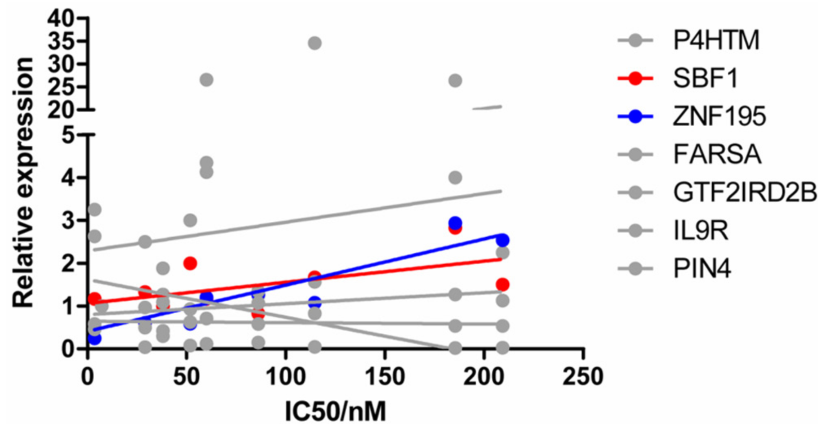

Figure 3. The correlation between the expression of 7 positive genes and gemcitabine response in 10 HNSCC cell lines. The top seven positive genes responsible for gemcitabine response (P4HTM, SBF1, ZNF195, FARSA, GTF2IRD2B, IL9R, PIN4) were validated by qPCR in the 10 HNSCC cell lines and only two genes (SBF1 and ZNF195) were significantly correlated to gemcitabine sensitivity.

Table 3. Independent validation in 11 cancer cell lines

cell	IC50/μM	relative expression	
		ZNF195	SBF
HCT116	10.00	3.83	0.29
GP5D	8.59	1.00	1.00
RKO	8.48	2.47	0.22
COLO205	6.25	2.01	0.98
SK-Mes-25	2.78	1.52	0.30
LOVO	2.52	1.26	0.18
HCT15	1.74	1.13	0.11
GP2D	1.20	0.84	0.19
OVCAR-3	0.37	0.64	0.22
SK-Mes-28	0.13	0.63	0.81
BPH-1	0.07	0.36	0.13
MG63	0.0005	0.26	0.30

ZNF195 expression was positively correlated with gemcitabine sensitivity (Pearson R = 0.83, p = 0.0008), whereas SBF1 expression was not in good correlation between gene expression and gemcitabine sensitivity (Pearson R = 0.35, p = 0.27). Therefore, ZNF195 is a potent biomarker for gemcitabine sensitivity in various types of cancer.

Discussion

Gemcitabine is a potential chemotherapy drug for HNSCC treatment, however, the poor or partial response of HNSCC patients to gemcitabine demonstrated the urgent need for gemcitabine biomarkers to improve the therapy.

In present work, we employed 10 HNSCC cell lines to figure out the biomarkers for gemcitabine sensitivity prediction. The sensitivities of these 10 cell lines to gemcitabine were distributed by nearly normal fashion, therefore these cell lines were suitable for gemcitabine biomarkers searching. Then the basal expression of these cell lines was investigated by DNA microarray. And the correlation between gemcitabine response (IC50 dose) and gene expression was investigated by Pearson correlation and FDR estimation. Those genes with Pearson R > 0.85 and FDR < 0.1% were denoted as positive genes responsible for gemcitabine sensitivity. Among the top 20 positive genes, RRM1 was predicted to be a true positive gene responsible for gemcitabine sensitivity, which was in line with previous studies [16, 36-39]. This result suggested that our prediction based on Pearson correlation and FDR estimation was reliable to some extent.

Then the top seven positive genes related with gemcitabine response (P4HTM, SBF1, ZNF195, FARSA, GTF2IRD2B, IL9R, PIN4) were validated by qPCR in these 10 HNSCC cell lines. The results demonstrated only two genes (SBF1 and ZNF195) were significantly correlated to gemcitabine sensitivity.

SBF1, a pseudophosphatase of the myotubularin family, contains an N-terminal GEF homology domain that modulates its growth regulatory properties [40]. Its deficient in mice causes male infertility, impaired spermatogenesis, and azoospermia [41]. SBF1 mutation causes Charcot-Marie-Tooth disease type 4B3 [42]. It is proposed that SBF1 together with its analogue MTMR2 regulates the activity of phosphatidylinositol 3-kinase (PI3K) and phosphatidylinositol 3-phosphate (PI3P) pathway [43, 44]. As PI3K pathway is well known signaling vital for cell growth and survival and closely correlated with drug sensitivity [45-47], hence it is

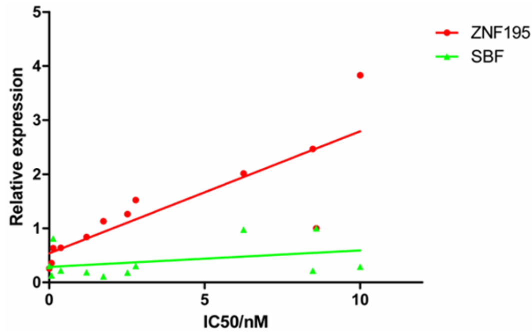

Figure 4. Independent validation by qPCR in 11 cancer cell lines. ZNF195 and SBF1 expression was investigated and tried to correlated to gemcitabine response in 11 cancer cell lines, while only ZNF195 was expression-correlated to gemcitabine response.

not surprising that expression of SBF1, regulator of PI3K activity, was correlated with response chemotherapy drug. Although SBF1 was not closely correlated with gemcitabine response in our independent validation, it is the first time to demonstrate that SBF1 expression is potentially associated with HNSCC initiation or progression but not in the other types of cancer. This result may warrant further investigation in more cancer cell lines and tissues.

ZNF195 encodes a protein belonging to the Krueppel C2H2-type zinc-finger protein family. These family members are transcription factors that are implicated in a variety of cellular processes. ZNF195 is located near the centromeric border of chromosome 11p15.5, next to an imprinted domain that is associated with maternal-specific loss of heterozygosity in Wilms' tumors [48, 49]. There are few studies about the function of ZNF195, however, ZNF195 was implicated in several types of cancer. ZNF195 is found to be selectively expressed in cutaneous T-cell lymphoma (CTCL) compared to normal peripheral blood monocytes [50] and to be alternatively spliced in human embryonal carcinoma and embryonic stem cells [51]. In our work, ZNF195 expression was closely correlated to gemcitabine sensitivity in HNSCC and various types of cancer. The underlying mechanism by which ZNF195 expression regulates cancer cell response to gemcitabine deserves further demonstration.

Collectively, our work might provide potential biomarkers for gemcitabine sensitivity in HNSCC and various type of cancer.

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Disclosure of conflict of interest

We have no conflict of interest to declare.

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Supplementary Table 1. Primers for qPCR validation

Gene	Forward	Reverse
Actb	CACCATGTACCCTGGCATT	GTACTTGCGCTCAGGAGGAG
P4HTM	CCGAACCCTCAGCCTCAAG	GATGGATGATGAGCCGACACT
SBF1	GCTCGCCGATGCCTGTAG	CAGGATGGGCACATAGGTGAA
ZNF195	ATGACTCTGTTGACGTTCAGGG	TGAGACCAACGGAGAACAAGT
FARSA	GACCAGCACGACACCTTCTTC	CTGAGAGTGGGTCCGCTTGA
GTF2IRD2B	CTCTGTTCAGCTCCCCGTTCT	GGACCGTGTTGCATTGCA
IL9R	GGAGACCCAGCAAGGAGTT	GACAGAAGGGAGCAACATGC
PIN4	GATGCGGCTTTCAGGCATT	CGCTCCAGTTGCCGTACAA