Author's response to reviews

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Version: 2 Date: 19 January 2006

Author's response to reviews: see over
Identification of genes specific to cisplatin resistance in human oral squamous cell carcinoma cell line

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Abstract

Background

Cisplatin is widely used for chemotherapy of head and neck squamous cell carcinoma. However, the molecular mechanism of resistance to cisplatin is still unclear. The aim of this study was to identify the gene expression profile of squamous cell carcinoma related to cisplatin resistance.

Methods

A cisplatin-resistant cell line, Tca/cisplatin, was established from a cisplatin-sensitive cell line, Tca8113, which was derived from moderate-differentiation tongue squamous cell carcinoma. Global gene expression analysis was performed on this cisplatin-resistant squamous cell carcinoma cell line and its sensitive parental cell line using Affymetrix HU95Av2 microarray. Four related genes were chosen for validation of the genechip analysis results.

Results

Resistance fold of Tca/cisplatin to cisplatin was 8.3-fold greater than Tca8113. Sixty-three genes were identified, among which 38 genes were up-regulated whereas 25 down-regulated in abundance when compared cisplatin-resistant cells with its sensitive parent. Among those well-established mechanisms for cisplatin resistance, some new candidate genes such as RECQL in DNA repair and MAP2K6 in MAP pathway were identified to be related to cisplatin resistance while others such as P-gp and GST-Pi were found unrelated. Furthermore, the up-regulated CCND1 and down-regulated CCND3 expressions were considered to be significantly related to cisplatin resistance.

Conclusions

The Tca/cisplatin cell line is useful in identifying the candidates responsible for the mechanism of cisplatin-resistance. The data from this study provided a profound clue to screen candidate targets for early diagnosis and intervention of cisplatin-resistance.

Key words: neoplasm; carcinoma; squamous cell; head and neck; microarray; cisplatin; drug resistance; cell line; gene expression.
Background

Head and neck squamous cell carcinoma (HNSCC) is a major public problem, frequently associated with devastating functional and cosmetic consequences in the patients. It is estimated that there were more than 500,000 new cases worldwide every year [1] and two thirds of patients present with locally advanced lesions and/or regional lymph node involvement. The benefit of chemotherapy on patients with advanced head and neck squamous cell carcinomas has been demonstrated by recent meta-analyses of randomized studies in several aspects, such as producing promising effects in reducing the rate of development of distant metastasis, improving survival rate and preserving organ function when combined with or without loco-regional treatment. [2] Cisplatin is one of the most potent chemotherapeutic agents known, exerting its cytotoxic action through interaction with DNA to form intrastrand crosslink adducts. [3] However, the DNA damage-mediated therapeutically beneficial apoptosis derived from cisplatin-induced DNA damage can be attenuated and the resistance that ensues is a major limitation of cisplatin-based chemotherapy. It is believed that the molecular signature defining the cisplatin resistant phenotype varies between tumors. Till now there have few genome wide analyses been performed in HNSCC, especially in oral squamous cell carcinoma, even though some analyses of gastric cancer cells, colon cancer cells and ovarian cancer cells were reported recently. [4-6] We established a cisplatin-resistant cell line, Tca/cisplatin, from a cisplatin-sensitive cell line and applied the Affymetrix HU95Av2 Array to analyze the different expressed gene pattern between this resistant cell line and its sensitive parental counterpart, aiming to identify cisplatin-resistant associated genes in this subtype of HNSCC.

Methods

Establishment of Cisplatin-Resistant Cells
Tca/cisplatin, a variant derived from Tca8113, was obtained by repeated exposure of cisplatin through gradually increasing concentrations that initiated at 0.3mg/L and ended at 3mg/L to acquire resistance to cisplatin. After 48 hours of treatment with different concentrations of cisplatin, survival rate of both cell lines was determined by MTT and Annexin V-FITC /PI Apoptosis Detection assays and repeated for 6 times. MTT assay was performed according to standard protocol in which absorbance of each well was read at 570nm on ELISA XL (BIOHIT, BP800). Survival rate was calculated
as the ratio of the absorbance of tested well to the absorbance of blank well, while inhibition rate (IR) was calculated as (the absorbance of blank well – the absorbance of tested well) / the absorbance of blank well × 100%. Annexin V-FITC/PI Apoptosis Detection (BD Pharmingen, USA) was used to further identify the difference of apoptosis induced by cisplatin following the protocol provided by the manufacturer. Results were analyzed with Cell Quest (Becton Dickinson).

**RNA Preparation and Gene Expression Analysis**

Gene expression profiles of the Tca/cisplatin and Tca8113 cell lines were analyzed on HG-U95Av2 Affymetrix oligonucleotide arrays (Affymetrix, Santa Clara, CA) containing 12,626 probe sets for human genes according to our previous study.[7] Comparison of gene expression profiles was made between Tca/cisplatin and Tca8113 cell lines, with Tca8113 cells as baseline using GeneChip Suite 5.0 software (Affymetrix). All of the genes represented on the GeneChip were globally normalized and scaled to a signal intensity of 500. Fold changes were calculated by comparing transcripts between the parent and the acquired drug resistant cell lines. In Microarray Analysis Suite software (Affymetrix), Wilcoxon’s test was used to generate detected (present or absent) and changed (increased or decreased) calls, from which it was statistically determined whether a transcript was expressed or not, and whether it was relatively increased, decreased, or unchanged. After being filtered through a “present” call ($P<$0.05), a transcript was considered different expressed if it was satisfied both of the following conditions: 1) by fold change, transcripts increased or decreased >2 fold; 2) by one-sided Wilcoxon’s rank test, $P<$0.003 or $P>$0.997.

**Semiquantitative RT-PCR**

Four genes CCND1, CCND3, P-gp and GST-pi were selected for semiquantitative RT-PCR (Life Technologies, Inc. Rockville, MD) to validate the results of genechip analysis according to our previous study. [8] The primer sets and product length were as follows: CCND1: product of 886 bp, up stream: AGA AAG CTT ATG GAA CAC CAG CTC CTG TG, down stream: GAG TCT AGA TCA GAT GTC CAC GTC CCG CAC GTC; CCND3: product of 898bp, up stream: AGA AAG CTT ATG GAG CTG CTG TGT TGC GAA G, down stream : GAG TCT AGA CTA CAG GTG TAT GGC TGT GAC A, GST-Pi: product of 159bp, up stream: TCC ACG GTC ACC ACC TCC TCC TC, down stream: TTT CGC CGC CGC AGT CT; P-gp: product of 157bp, up stream: CCC ATC ATT GCA ATA GCA GG, down stream: GTTCAA ACT TCT GCT CCT GA. PCR was fulfilled as follows: initial denaturation at 94°C for 5 minutes followed by 27 cycles of 94°C for 30s, 58°C for 30s, 72°C for 45s, and ended with an elongation at 72°C for 7 minutes. The products were all electrophoresed on 1% agarose gel and the
optical density of each band was analyzed with TotalLab software (v2.01).

**Western Blot Analysis**

To investigate the correspondence between mRNA and protein of above selected genes we further performed Western blot analysis of Tca/cisplatin and Tca8113 cell line. Total protein was extracted from cells using lysis buffer (M-Per, Pierce). First antibodies were: mouse anti-human monoclonal antibody cyclin D1 (Dakocytomation), mouse anti-human monoclonal antibody cyclin D3 (Abcam), mouse anti-human monoclonal antibody Pgp (Dakocytomation), mouse anti-human monoclonal antibody GST-Pi (Dakocytomation). Signals were visualized with Supersignal West Pico Chemiluminescent Substrate (Pierce). Result was analyzed with TotalLab software (v2.01).

**Statistics**

Student’s t test was used to determine the statistical differences between these two cell lines in semiquantitative RT-PCR and Western blot assays. A value of $P<0.05$ was considered as statistical significance.

**Results**

**Cisplatin-resistant Phenotype of Tca/cisplatin Cell line**

Tca/cisplatin cell line was obtained by stepwise selection from its sensitive parental cell line. At the beginning of induction, growth of cells was severely suppressed. However, at the end of induction, the derived cells grew at a significantly high proliferation rate. After further maintained in cisplatin-free RPMI1640 supplemented with 10% fetal bovine serum till 60 passages, the resistant cell exhibited with stable biological characteristics, among which the population doubling time was 29.9 hours, while that of the parental cell was 38.8 hours. The drug resistance had been stable for two years without maintenance dosing of cisplatin.

The coincident results in survival rates between MTT assay and Annexin-V and PI double staining were obtained. Although cisplatin treatment resulted in a dose-dependent growth inhibition in both cell lines, the survival rate decreased much more significantly in Tca8113 cells than in Tca/cisplatin cells as the cisplatin concentration increased. The IC$_{50}$ (drug concentration resulting in 50% growth inhibition) was 8.3 mg/L and 1 mg/L in the resistant and sensitive cell line, respectively.

**Different Gene Expression Profiles between Tca/cisplatin and Tca8113**

Affymetrix HG-U95Av2 genechip contained 12626 probe sets and most of them were previously characterized in term of function or disease association. As shown in Table 1, a total of 63 probe sets were selected as having significant fold-changes and $P$ value...
changes, among which 38 genes were up-regulated and 25 genes down-regulated in the Tca/cisplatin cell line. Most of the different expressed genes were involved in the regulation of cell proliferation and metabolism. These 63 genes were primarily classified according to their potential function (based on the listing of Gene Oncology terms) into several groups as shown in Table 1.

**Validation of Genechip Results**

Four genes were selected, named CCND1, CCND3, GST-Pi and P-gp, to verify the results of microarray by semi-quantitative RT-PCR and by Western blot at the same time. CCND1 was increased in Tca/cisplatin cells, CCND3 was decreased in Tca/cisplatin cells, GST-Pi was over-expressed in both cells and P-gp was absent. Figure1 showed the results of three separate experiments, in which up-expressed CCND1 and down-expressed CCND3 in the cisplatin resistant cells were found significantly different from those in the parental sensitive cells on mRNA and protein levels \( (P<0.05, \) student’s \( t \)-test). While GST-Pi did not show difference in gene expression in both two cells \( (P>0.05, \) Student’s \( t \)-test) and P-gp was absent in both cells. These four selected genes demonstrated excellent agreement with the results from the microarray analysis.

**Discussion**

It is clear from our results that cisplatin-resistant cell line, Tca/cisplatin, has a genome-wide genetic expression profile that differs from primary Tca8113 cell line. Having determined the existence of such differences, the next question is to what extent are these gene expression alterations related to cisplatin-resistance. Or whether there is genetic expression profile heterogeneity among malignant tumors of a particular histopathologic grade and chemotherapeutic agent resistant potential? Future studies are needed to directly compare gene expression profiles between different samples of the same stage to confirm this hypothesis. Analysis of cell lines using microarray also revealed several novel differentially expressed genes, which have not been reported to be related to chemotherapeutic agents resistance, for example, GRP58, FLJ12089, SPINT-2, FOSL1, MRPS27, PGK-1, MET, CCNC, GAS1, TFDP2, MAPK10/JNK3, WEE1, RPA1 genes etc. [9-11] Further validation and detailed mechanism of these genes in conferring the phenotype of cisplatin resistance in the malignancies needed more extensive investigation. The global molecular regulatory network would also shed a light on our understanding of the mechanism of pathogenesis of chemotherapeutic agent resistance and potential strategies of its reversal. [11, 12] Considering the same genetic background between Tca/cisplatin and Tca8113 cells, we
suppose that the differently expressed 63 genes detected in our microarray analysis related to cisplatin resistance. The down-regulated genes included those involved in cell cycle arrest, cell proliferation regulation and nucleic acid binding protein metabolism, while the up-regulated genes mostly included those, such as cell cycle regulation gene (CCND1), oncogenes (c-syn, FGFR3, RAB31), and genes involved in small molecule non-selective transport (ITPR1), protein synthesis (GARs), nucleobase DNA repair (RECQ1) and transcriptions (Id1, Id2). The up-regulation of these genes might result in accelerating cell cycle, increasing proliferation, enhancing DNA metabolism and synthesis, altering molecular transport and elevating transcription activity, as that demonstrated as a shorter population doubling time in Tca/cisplatin (29.9 hours) than its parental cell (38.8 hours).

Two in-depth studied mechanisms are the decrease of intracellular drug concentration caused by overexpression of P-gp and the enhancement of cellular detoxification resulted from an increase of GST-Pi. [13-15] However, in our study, no difference in the expression level of P-gp and GST-Pi were detected in genechip analysis. Due to our samples have been cultured in cisplatin – free conditions for near 2 years, no different expression of Pgp and GST-Pi may reflect their characteristics as transient drug resistant genes. An enhanced rate of DNA repair would attenuate the apoptotic process induced by formation of DNA adducts of cisplatin, and that had been demonstrated in several studies in murine and human tumor cell lines. [16-20] In our microarray analysis, overexpression of RECQL, a DNA helicase involved in various types of DNA repair including mismatch repair, nucleotide excision repair and direct repair, was identified in cisplatin resistant cells, though this is the first identification of its association with cisplatin resistance. [21]

Cell cycle deregulation has been related to acquisition of drug resistance. Overexpression of CCND1 has been proposed to promote resistance to cisplatin in cancer cells of breast, pancreas, colon and lung. [21-24] Our microarray analysis showed an interesting altered pattern of expression of cell cycle genes CCND1 and CCND3: CCND1 was up-regulated while CCND3 down-regulated. The D-type cyclins, responsible for initiating cell cycle progression from quiescent G0 to G1, were expressed in an overlapping and redundant manner, for example in human mammary epithelial cells CCND1 expression peaks in G1 and declines before the S phase, while CCND3 expression rises later in G1 and remains elevated in S phase. [25] CCND1 is preferentially associated with cyclin-dependent kinases 4 and 6 to inactivate the retinoblastoma tumor suppressor protein via phosphorylation, thereby overcoming the RB-mediated blockade of G1 and facilitating G1-S progression. So that it was
reasonable to consider that the inverse expressions of CCND1 and CCND3 might participate in the cisplatin resistance of squamous cell carcinoma.

We reported here 63 probe sets with average 2-fold different expression ratio in cisplatin resistant and sensitive cell line, and attributed this probe list to cisplatin resistant traits as both cell lines were derived of the same origin: Tca8113 was established from primary human tongue squamous cell carcinoma and Tca/cisplatin was screened out from Tca8113. We have selected only those genes whose expression was significant changed and satisfied at least two conditions. Due to cutoff point of conditions is arbitrary and some important genes whose expression changed less than the two factors would be missed. There might be some false negative or false positive resistance-related genes in our probe list.

The measurement of gene expression can provide information on regulatory mechanisms, biochemical pathways, cellular control mechanisms and potential targets for intervention and therapy in a variety of disease states. [10] Our results support the hypothesis that multiple specific genes contribute to the development of resistance to cisplatin of squamous cell carcinoma. Microarray analysis of the model system for differentially expressed genes involved in cisplatin resistance of squamous cell carcinoma has revealed a variety of specific genes, including putative common drug resistance-related genes, which provides a basis for rationally determining which pathways are appropriate for further study and which molecular targets are potential targets for gene therapy. [11, 12] These findings will provide new insights into further exploring the complicated molecular events of drug resistance. Studies of molecular mechanism regulating these changes would help to identify prognostic marker and treatment targets for HNSCC chemotherapy.

Conclusions

The Tca/cisplatin cell line is useful in identifying the candidates responsible for the mechanism of cisplatin-resistance. 63 genes related to cisplatin-resistant of oral squamous cell carcinoma were identified. Of them, decrease in cell cycle arrest genes and increase in oncogenes, cell cycle regulation gene and genes involved in metabolism and synthesis, led to accelerating cell cycle and proliferating rate in resistant cells. CCND1 and CCND3 seemed to closely involve the cisplatin resistance of oral squamous cell carcinoma. The data from this study provided a profound clue to screen candidate targets for early diagnosis and intervention of cisplatin-resistance.
Acknowledgements

This study was supported by National Natural Science Foundation of China of key project (Grant No. 30330580). And was supported by National Natural Science Foundation of China (Grant No. 30171014, 30300388).
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