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An increase of cytochrome C oxidase mediated disruption of gemcitabine incorporation into DNA in a resistant KB clone

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ABSTRACT

Mechanistic aberrations leading to Gemcitabine (2′,2′-dFdCyd,2,2-difluorodeoxycytidine, Gem) resistance may include alteration in its transport, metabolism and incorporation into DNA. To explore the mechanism of Gem resistance, the restriction fragment differential display PCR (RFDD-PCR) was employed to compare the mRNA expression patterns of KBGem (Gem resistant), KBHURs (hydroxyurea resistant) and KBwt (parental KB cell). Nine gene fragments were overexpressed specifically in the KBGem clone. Sequencing and BLAST results showed that three fragments represent cytochrome C oxidase (CCOX, respiration complex IV) subunit III (CCOX3). The cDNA microarray confirmed that the mRNAs of CCOX and ATP synthase subunits were upregulated in KBGem as compared to KBwt and KBHURs. The increase in CCOX1 protein and activity led to the increase of free ATP concentration, which is consistent with the gene expression profile of KBGem. Furthermore, the sensitivity to Gem could be reversed by sodium azide, a CCOX inhibitor. Following the treatment of sodium azide, the cellular accumulation of [^{3}H]-Gem increased in a dose (of azide)-dependent manner, which is associated with increase of [^{3}H]-Gem incorporation into DNA in KBGem. In summary, an increase of CCOX activity and free ATP level may reduce the transport, metabolism and DNA incorporation of Gem, resulting in Gem resistance.

1. Introduction

Gemcitabine (2′,2′-dFdCyd,2,2-difluorodeoxycytidine, Gem), a deoxycytidine analogue, is widely used in the treatment of hematological malignancies and solid tumors [1]. Gem is sequentially phosphorylated by deoxycytidine kinase (dCK), dCMP kinase (dCMPK) and nucleoside diphosphate kinase (NDPK), which is then further metabolized to form dFdCDP and dFdCTP [2,3]. As a competitor of dCTP, dFdCTP is incorporated into DNA during replication causing chain termination [4,5]. Drug resistance is the major limiting factor for Gem based therapy. The resistance to Gem can be caused

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Abbreviations: Gem, 2′,2′-dFdCyd,2,2-difluorodeoxycytidine, Gemcitabine; dCK, deoxycytidine kinase; dCMPK, dCMP kinase; NDPK, nucleoside diphosphate kinase; CK, cytidylate kinase; CCOX, cytochrome C oxidase; ENT, equilibrative nucleoside transporter; CNT, concentrative nucleoside transporter; OAT, organic anion transporter; OCT, organic cation transporter; PEPT, peptide transporter; MRP, multi-drug resistance protein; KBGem, gemcitabine drug resistant KB clone; KBHURs, hydroxyurea drug resistant KB clone; KBwt, parental KB cell; hRR, human ribonucleotide reductase; hRRM1, human ribonucleotide reductase large subunit M1; hRRM2, human ribonucleotide reductase small subunit M2; p53R2, p53 dependent human ribonucleotide reductase small subunit R2 (M2B); dNTP, deoxyribonucleoside triphosphate; NDP, ribonucleoside diphosphate; RFDD-PCR, restriction fragment differential display PCR

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by a number of factors affecting its transport, metabolism and incorporation into DNA [6]. These factors include a deficiency in the equilibrative/concentrative nucleoside transporter protein [7–10], an increased nucleotidase that catalyze the conversion of nucleotide back to nucleoside [11], a loss of dCK expression [12,13] and an increase in hRR activity [14,15]. It has been suggested that the increase in hRR activity will lead to expand the dCTP pool, which may then competitively inhibit the incorporation of dFdCTP into DNA during replication [15]. However, the mechanism cannot completely explain how the increase in hRRM2 leads to Gem resistance. A 10-fold increase in hRRM2, resulting in a 2-fold increase of the dCTP pool increase, cannot fully explain the 100-fold increase in resistance observed in a Gem resistant clone (KBGem). It implies that as yet unidentified factor may act synergistically with overexpressed hRRM2 to cause Gem resistance.

Cytochrome *C* oxidase (CCOX, complex IV, EC:1.9.3.1.), the terminal enzyme in the respiratory chain, is located in the inner membrane of mitochondria [16]. It reduces oxygen to water and pumps protons across the inner mitochondrial membrane. This proton gradient is used by ATP synthase to generate ATP [17]. CCOX contains 13 subunits per monomer. Subunits I, II and III are responsible for the enzyme's catalytic activity, and are encoded by the mitochondrial genome [18]. Nuclear genes encode the other 10 subunits of CCOX [19]. Under the physiological condition, CCOX has been shown to be the rate-limiting enzyme of oxidation [16]. The activity of CCOX is allosterically regulated by the ATP/ADP ratio [17,20]. Azide and cyanide exhibit specific inhibitory effects on the mitochondrial CCOX [21]. Alteration of CCOX subunits has been reported in adriamycin, doxorubincin and cisplatin resistant clones [22,23]. Yet, how CCOX affects resistance to nucleoside analogues is not known.

In this report, we show that CCOX subunit is overexpressed in a Gem resistant clone (KBGem) overexpressing hRRM2. Microarray results further show that many genes of the respiration complex IV (cytochrome *C* oxidase) and complex V (ATP synthase) families were upregulated in KBGem. The increase in CCOX activity and free ATP level was also confirmed in KBGem. Furthermore, sodium azide, a CCOX inhibitor, could revert the Gem resistance in KBGem. Thus, increasing the CCOX expression and activity may alter Gem's efflux and metabolism, contributing to Gem resistance.

2. Material and methods

2.1. Cell culture and Gem resistant clones' selection

The human oropharyngeal epidermal carcinoma cell line KB was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% Penicillin–Streptomycin. The selection of Gem resistant clones was based on KB cell step-wise exposure to increased concentrations of Gem [15]. The Gem resistant clones were selected and maintained in the presence of 8 μM Gem.

2.2. Western blots

Each cell lysate (40 μg) was separated and transferred to a PVDF membrane. The transferred PVDF membranes were incubated in blocking buffer (1% I-block™ reagent and 0.1% Tween-20) with the primary antibody (1:200 dilution) for 45 min at room temperature, washed with blocking buffer, and then incubated with 1% I-block™ blocking buffer with alkaline phosphatase conjugated secondary antibody (1:2000 dilution) for 30–60 min. This was followed by sequential washes with 0.5% I-block™ buffer and assay buffer (200 mM Tris–HCl, 10 mM $MgCl_2$). Then a thin layer of CSPD Ready-to-Use substrate solution (Applied Biosystems, Foster City, CA) was transferred over the membrane. After a 5-min incubation, the membrane was exposed to X-ray film for 3 min.

2.3. Restriction fragment differential display PCR (RFDD-PCR)

The analysis RFDD-PCR was carried out as described in detail in the protocol from the display PROFILE kit (Qbiogene, Irvine, CA). Briefly, the total mRNA was extracted from KBwt, KBGem and KBHURs by using Oligotex mRNA Midi Kit (QIAGEN, Valencia, CA). Total mRNA was reverse-transcribed using a randomized octamer primer to synthesize cDNA. Then, 10 μl cDNA was digested with TaqI endonuclease at 65 °C for 2 h. The RFDD-PCR template was completed by ligating the digest to adaptor mixture containing an extension protection group at 37 °C for 3 h using T4 DNA ligase.

The template was PCR amplified using a zero-extension primer complementary to the EP adaptor in combination with a three-extension primer recognizing the standard adaptor and the three nucleotides adjacent to the TaqI site. The zero-extension primer was fluorescently labeled with Cy5. All PCR reactions were carried out in a 20 μl volume using 0.2 μl template and standard concentration of dNTPs and primers. For each template, 32 PCR reactions using the Cy5-labeled zero-extension primer in combination with the 32 different three-extension primers (NNA/G or NNC/T) were performed. The PCR products were separated on a standard 6% polyacrylamide sequencing gel. Bands corresponding to those indicated by the fluorescence-based analysis to be differentially expressed were identified on the autoradiogram. The dried gel was lined up with markings on the film and the fragments were excised from the gel. The gene fragments were re-amplified using the same PCR conditions and primers as in the initial PCR reaction. The PCR re-amplified products were sequenced by City of Hope co-facility.

2.4. Cytochrome C oxidase activity assay

The cytochrome *C* oxidase assay kit (Product Code: CYTOC-OX1) was purchased from SIGMA (St Louis, MO). Briefly, assays contained 400 μg fresh cell lysates and were performed at room temperature in 1.1 ml reaction volumes. The assay involved the addition of 0.01 mM ferrocytochrome c in assay buffer (10 mM Tris–HCl, pH 7.0, 120 mM KCl). The activity was calculated from the rate of decrease in absorbance of ferrocytochrome c at 550 nm ($\Delta\varepsilon^{mM} = 21.84$) and results were normalized by an internal control provided by the kit.

2.5. Microarray analyses

The mRNA isolation, cRNA preparation, microarray hybridization and data analysis have been described in our previous publication [24,25]. The HG-U95Av2 microarray set from Affymetrix (Santa Clara, CA) was employed to analyze the gene mRNA expression. The analyses were performed at the Microarray Core Facility, Children's Hospital Los Angeles. The data was analyzed using Affymetrix Microarray Software Suite (v.4.0) and GeneSpring™ (v7.0) software (Silicon Genetics, Foster City, CA). The quality of hybridization was evaluated with internal control spots. Each chip was normalized with the standard protocol of the core facility. Spots representing housekeeping genes were further used to normalize the entire slide so that all slides could be compared directly. This microarray data has been deposited to Geo (http://www.ncbi.nih.gov/geo/). The access number of KBwt, KBGem and KBHURs are GSM69543, GSM69658 and GSM69660, respectively.

2.6. ATP concentration detection assay

The concentration of ATP was determined using the ENLITEN® ATP Assay system (Promega, Madison, WI). About 1×10^6 cells were harvested and counted, and then suspended in 200 μl of 10% TCA solution on ice for 20 min. After centrifuging for 10 min, the supernatant was collected and diluted 1:100 in 50 mM Tris–HCl buffer (pH 8.0), and stored at −80 °C for detection. The reaction included 100 μl of rL/L reagent buffer and 20 μl of the diluted sample. Fresh standard ATP samples were prepared to measure the concentration of samples.

2.7. [^{3}H] labeled Gem and dCy cell uptake and DNA incorporation assay

About 1×10^6 cells were evenly seeded on 100 mm^2 cell culture dishes. To inhibit CCOX activity, the cells were incubated with 0, 7.5, 15, 30, 60 and 120 μM of sodium azide (Sigma) for 3 h, and then incubated with [^{3}H] labeled Gem (Moravek Biochemicals, Brea, CA) (3.5 μCi/ml, 0.25 nmol/ml) or [^{3}H] deoxycytidine (dCy, Moravek Biochemicals, Brea) (3.5 μCi/ml, 0.25 nmol/ml) for another 3 h. Cells were washed twice and harvested using 0.5% trypsine. The incubation medium was collected and measured with a scintillation counter as a control. DNA was extracted from 8×10^5 cells using QIAamp® DNA Mini extraction kit (QIAGEN). The DNA concentrations were measured and detected by 280 and 260 nm wavelengths. The cell uptake and DNA incorporation of [^{3}H]-Gem/dCy were measured with one-fifth cell pellets (about 2×10^5 cells) and 50 μg of DNA samples by liquid scintillation counter, respectively. The [^{3}H]-Gem/dCy DNA incorporation was adjusted by DNA concentration.

2.8. Statistics

Data was collected using an MS-Excel spreadsheet. Data was analyzed using the JMP Statistical Discovery Software version 6.0 (SAS Institute, Cary, NC). Comparisons for continuous data were performed using *t*-tests for independent means or one-way analyses of variance. Error-bars are instead of standard deviation. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Characterization of the Gem resistant cell line KBGem

To explore the mechanisms of Gem resistance, we isolated a Gem resistant clone (KBGem) via step-wise selection. KBGem was maintained in 8 μM of Gem (IC_{50} of parental KB cell is 0.3 μM) [15]. The hydroxyurea drug resistant clone KBHURs and the parental KB cells (KBwt) were used as controls. KBHURs was also selected via step-wise selection. Previously, it was shown that KBHURs overexpress hRRM2 at mRNA and protein levels, and exhibits cross-resistance to Gem phenotype [15]. The characteristics of KBwt, KBGem and KBHURS clones are summarized in Table 1. Using an upgraded version

Table 1 – Characterization of drug resistant clones and parental cell

	KBwt	KBGem	KBHURs
Relative amounts of mRNA[a]			
hRRM1	1.0	1.2	1.5
hRRM2[b]	1.0	29.3	32.0
p53R2	1.0	0.9	1.0
Relative amounts of protein[c]			
hRRM1	1.0	1.0	1.1
hRRM2[d]	1.0	9.0	10.0
p53R2	1.0	1.0	1.0
Relative RR activity[e]	1.0	2.3	6.7
dCTP pool (pmol/1,000,000 cell)[e]	0.90 ± 0.58	1.80 ± 0.95	13.4 ± 6.73

[a] Determined from phosphorimaging measurements of signal hybridizing with corresponding probe with RNA extracted from KBwt, KBGem and KBHURs clones. Data was normalized with a signal value of GAPDH.
[b] mRNA of hRRM2 increases significantly in KBGem and KBHURs (grouped *t*-test, $n = 3$, $p < 0.01$).
[c] Western blot was employed to evaluate the protein level of KBwt and KBGem clones. The results were normalized with a signal value of α-tubulin.
[d] Protein levels of hRRM2 increase significantly in KBGem and KBHURs (grouped *t*-test, $n = 3$, $p < 0.01$).
[e] RR activity and dCTP pool assay were described in our previous paper [44].

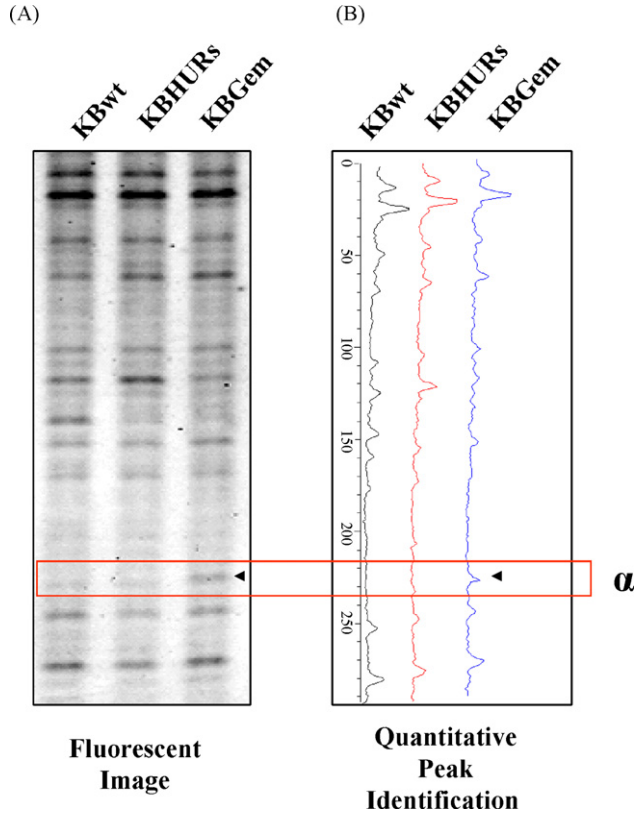

Fig. 1 – Fluorescent RFDD-PCR analysis for KBwt, KBHURs and KBGem. The left panel (A) shows subsequent fluorescent analysis and the identification of the bands for gel excision. The right panel (B) shows identification of the high expression fragments using ImageQuant 5.2 software. The fragment (α) is E12G5-5 overexpressing in KBGem only.

of phosphorimage scanner and a quantitative software (ImageQuant 5.2), a greater increase of hRRM2 was detected in KBGem and KBHURs than we previously reported [15]. KBGem and KBHURs express 29.3- and 32.0-fold higher level of hRRM2 mRNA with 9.0- and 10.0-fold increase in hRRM2 protein, respectively. It led to approximately 2.3- and 6.7-fold increase in RR activity, which, in turn, caused 2- and 14-fold expansion of dCTP pool in KBGem and KBHURs, respectively. These results indicated that both KBGem and KBHURs over-expressed hRRM2 in similar range, but discrepancy of gem resistance could be based on different molecular mechanisms.

3.2. *Identification of highly expressed gene fragments in KBGem*

To investigate as yet unidentified factors contributing to Gem resistance in KBGem, RFDD-PCR was employed to compare transcript patterns among the above clones. The Cy5-labeled RFDD-PCR products were separated through a polyacrylamide gel and the results pattern was analyzed with ImageQuant 5.2 software. The difference in the expression level was quantitated by comparing peak areas (Fig. 1A and B). Only the fragments with at least a five-fold increase in expression were considered for the analysis. In total, nine gene fragments were identified with a higher expression in KBGem compared to KBwt and KBHURs. Those nine fragments were re-harvested and re-amplified using corresponding primers. The sequences of the fragments were used to identify genes using Genebank's BLAST program. As shown in Table 2, nucleotide–nucleotide BLAST (blastn) alignments indicated that five of the nine fragments (E12G5-5, E33G5, E33G5L, E34G5L and E51G5) exhibit a high-degree of similarity to mitochondrion. E32G5, E48G5 and E9G5-10 were identified as poly (A) binding protein, thymosin beta 10 and TNF receptor-associated factor 4 isoform 1, respectively. Searches performed using translated sequence as query against the protein sequence database

Table 2 – Identification of fragments of RFDD-PCR

Fragments	BLAST[a] search results			
	blastn[b]		blastx[c]	
	Gene	Identities (%)	Protein	Identities (%)
E12G5-5	Mitochondrion	99	CCOX III	97
E32G5	Poly(A) binding protein	100	Poly(A) binding protein	97
E33G5	Mitochondrion	96	NS[d]	
E33G5L	Mitochondrion	99	CCOX III	89
E33G5S	NS[d]	–	NS[d]	–
E34G5L	Mitochondrion	100	CCOX III	95
E48G5	1-Thymosin beta 10	92	NS[d]	–
	2- Migration-inducing protein 12	92		
E51G5	Mitochondrion	94	NADH dehydrogenase subunit 1	84
E9G5-10	TNF receptor-associated factor 4 isoform 1	98	1-TNF receptor-associated factor 4 isoform 1	100
			2-Cystein rich domain associated to RING and TRAF protein	100

[a] BLAST: Basic local alignment search tool (http://www.ncbi.nlm.nih.gov/BLAST/).
[b] Nucleotide-nucleotide BLAST.
[c] Translated query vs. protein database, Nucleotide-protein BLAST.
[d] NS: No significance found.

Table 3 – Expression profile of Respiration complexes' genes

Systematic	KB	KBGem[a]		KBHURs[b]		Gene	Genbank
	Raw	Raw	Normalized	Raw	Normalized		
Complex I, NADH dehydrogenase (ubiquinone)							
40546_s_at	1740.7	2565	1.53	1468.6	0.78	NDUFA2	AF047185
32752_at	6893.2	15895.7	2.39	4690.1	0.63	NDUFA7	W72440
32774_at	1413.7	2298.1	1.68	1443.5	0.95	NDUFB8	AI541050
36169_at	24882.6	26412.8	1.10	23343.6	0.87	NDUFA1	N47307
38060_at	8974	21215.9	2.45	7122.8	0.74	NDUFS5	AI541336
38695_at	1262.3	1559.7	1.28	1607.4	1.18	NDUFS4	AA203303
32738_at	1253.3	1039.5	0.86	1029.3	0.76	NDUFS2	AF050640
36205_at	642.9	1333.4	2.15	839.9	1.21	NDUFA9	L04490
Complex II, succinate dehydrogenase							
34826_at	3995.4	3796.1	0.98	5594.1	1.30	SDHA	L21936
35751_at	832.6	1421.5	1.77	900	1.00	SDHB	U17886
Complex III, ubiquinol-cytochrome C reductase							
40854_at	2185.4	921.8	0.44	1544.3	0.66	UQCRC2	J04973
39427_at	6601.8	3222.3	0.51	6534.6	0.92	UQCRB	T79616
34400_at	17961.1	35961.6	2.07	14174.7	0.73	QP-C	AI540957
36104_at	4401.2	3315.5	0.78	3814.5	0.80	UQCRH	AA526497
38451_at	1403.1	3167.3	2.34	1119.2	0.74	UQCR	T58471
Complex IV, cytochrome C oxidase							
40427_at	1141.9	1033.6	0.94	1095.2	0.89	CCOX17	AA149486
41223_at	971.5	592.3	0.63	720.9	0.69	CCOX5A	M22760
39443_s_at	3051.7	6813	2.31	2091.3	0.64	CCOX5B	M19961
41206_r_at	2414.2	6303.9	2.70	1710.2	0.66	CCOX6A1	AI540925
36165_at	5659.5	4452.5	0.81	4633	0.76	CCOX6C	W51774
41760_at	2413.6	7305.7	3.13	2102.6	0.81	CCOX7A2	AA978033
36687_at	8131.6	9844	1.25	11017.2	1.26	CCOX7B	N50520
34381_at	8714.4	7479.8	0.89	9982.4	1.06	CCOX7C	AI708889
38080_at	15853.4	31564.1	2.06	9329.8	0.55	CCOX8	AI525665
38660_at	771.4	1045.6	1.40	695.6	0.84	CCOX6A2	F27891
40872_at	688	4391	6.61	823.5	1.11	CCOX6B	T57872
34723_at	340.8	338.6	1.03	259.2	0.70	CCOX11	U79270
Complex V, ATP synthase							
41357_at	3148.4	3838.3	1.26	5571.3	1.64	ATP5B	W27997
40115_at	12487.2	21879.4	1.81	3582.6	0.27	ATP5C1	D16562
37992_s_at	3008.9	3994.9	1.38	1305.1	0.40	ATP5D	AI436567
36107_at	1848.8	2120	1.19	1369.9	0.69	ATP5J	AA845575
37029_at	2344.2	2480.7	1.10	2489.2	0.98	ATP5O	X83218
34811_at	2370.8	3315.1	1.45	1659.1	0.65	ATP5G3	U09813
35760_at	1336.5	1387.6	1.08	2523.7	1.75	ATP5H	AF087135
38751_i_at	108199.1	133973.7	1.28	107770.2	0.92	ATP5I	AA426364
40134_at	1258.8	6149.1	5.06	1233.9	0.91	ATP5J2	AF047436

[a] Paired *t*-test, $p = 0.004$, KBGem compared with parental KB cell.
[b] Paired *t*-test, $p = 0.076$, KBHURs compared with parental KB cell.

(blastx) identified three gregments as cytochrome C oxidase subunit III (E12G5-5, E33G5L, E34G5L), and one fragment as NADH dehydrogenase subunit 1. It indicated that an increase of CCOX and other mitochondrial respiration complexes may be related to the Gem resistance phenotype of KBGem.

3.3. *Determination genes of the expression profile of respiratory complex subunits using cDNA microarray*

cDNA microarray analysis was used to further validate the expression pattern of the genes relating to CCOX function. Signals were normalized with an internal control chip. After normalization, numerous changed genes were detected in KBGem and KBHURs, but most of affected genes appeared to have little relevance to the Gem resistance mechanism (data not show). Because Affymetrix HG-U95Av2 microarray did not include mitochondrial genes, the expression of CCOX subunits I, II and III could not be detected. Here, by filtering the present signal (P) using Genespring software, valid signals of respiration complex subunits are shown (Table 3). The results showed that most of the genes in complex I (NADH dehydrogenase), complex IV (cytochrome C oxidase) and complex V (ATP synthase) were upregulated in KBGem (compared to KB, paired *t*-test, $p = 0.004$). Most of the listed genes did not show difference that is statistically significant between KBHURs and KBwt (paired *t*-test, $p = 0.076$). The NADH dehydrogenase subunit 1 that was identified as an upregulated gene via RFDD-PCR increased slightly in KBGem. Nevertheless, the above results indicate that most of the genes of the respiration complexes were increased in KBGem, which may be associated with Gem resistance.

3.4. Increase of CCOX subunits expression and activity in KBGem

To confirm the up-regulation of CCOX in Gem resistant cells, the expression level of CCOX subunits and CCOX activity were determined in KBGem. The Western blot results showed that the CCOX subunit 1 increased 3.6-fold in KBGem, but only 1.7-fold in KBHURs (Fig. 2A). The CCOX subunits 2 and 3 levels could not be similarly determined due to the in efficient antibodies. KBGem had a higher CCOX activity than KBwt and KBHURs, but a large variation between samples prevent us from determining statistical significance (Fig. 2B). As CCOX represents the terminal enzyme in the respiratory chain providing the proton gradient for ATP synthesis, the ATP concentration was also determined. KBGem exhibited approximately four-fold increase of ATP concentration in comparing to KBwt and KBHURs (Fig. 2C). The above findings confirmed that the increase in CCOX ATP synthase of the respiration complex is associated with Gem resistance phenotype in KBGem.

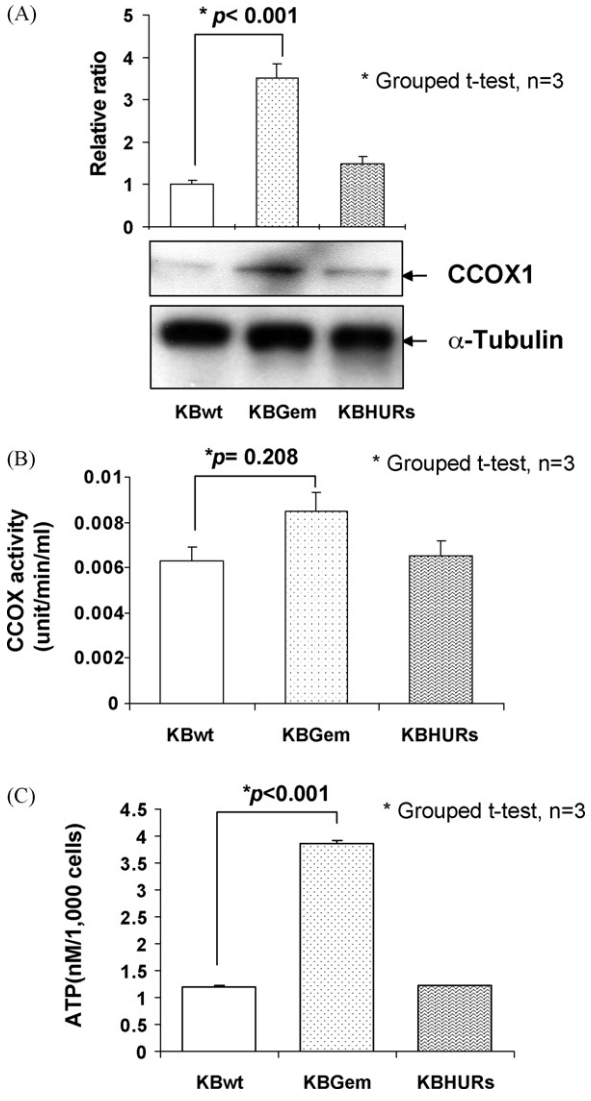

Fig. 2 – Increase of CCOX1 expression and activity in KBGem clone. (A) Western blot: The mouse antibody against CCOX subunit 1 was employed to determine protein levels in KBwt, KBGem and KBHURs clones. The α-tubulin was a loading control. Error bars are used instead of standard deviation. (B) *In vitro* CCOX activity assay: 100 μg of cell lysates extracted from each cell clone were used to evaluate the CCOX activity. The activity calculation was performed according to the parameter described in Section 2. (C) The free ATP was determined using the ENLITEN® ATP Assay system. Cell number was employed to normalize the ATP concentration.

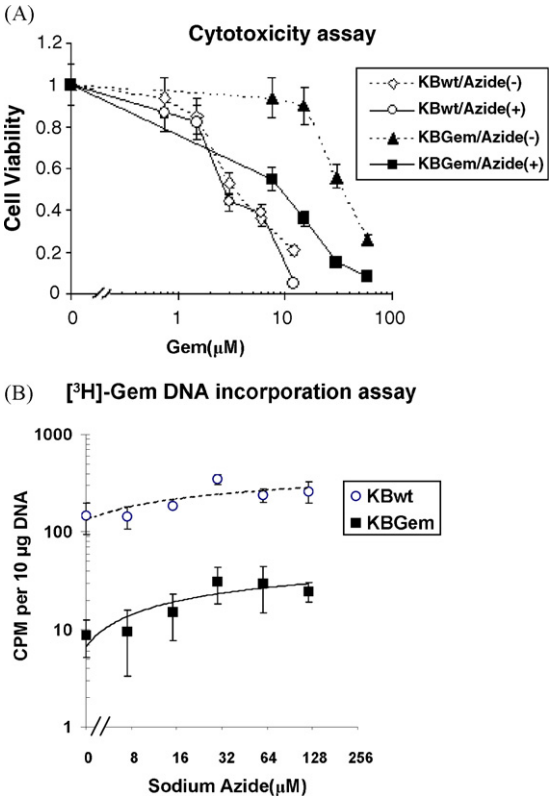

Fig. 3 – Determining Gem sensitivity and incorporation into DNA under treatment of azide. (A) Cytotoxicity assay: About 5000 cells/100 μl was seeded to a 96-well plate. Thirty micrometers of sodium azide was added to the azide treatment group. After incubating for 72 h, each well was added 20 μl of Aqueous One Solution Reagent (Promega, Madison WI) and then incubated for an additional 2 h. After incubation, each well read the o.d. at 490 nm. Cells' survival curve was normalized by corresponding control. (B) DNA incorporation assay: Cells were pre-treated with different dosages of sodium azide, CCOX specific inhibitor, for 3 h. Following incubation of [^{3}H] labeled Gem/cytidine (3.5 μCi/ml, 0.25 nmol/ml) for another 3 h, the cells were washed with PBS twice, and then trypsinized and harvested. Genomic DNAs were extracted and concentration determined. Fifty micrograms of extracted DNA was counted for tritium signal value.

3.5. *Reversal of Gem resistance by using CCOX inhibitor in KBGem*

The effect of azide on mitochondrial respiratory enzymes is limited to CCOX [26]. The treatment dose of sodium azide was based on a previous study [27]. Following the treatment with azide at 30 μM, the survivability of KBwt was little affected, but the IC_{50} of KBGem for Gem decreased from 33.2 to 15.3 μM (Fig. 3A). A similar co-treatment with azide did not significantly impact survivability of KBGem to SAHA (a HDAC inhibitor) and ps341 (a proteasome inhibitor) (data not shown). Above results indicated Azide could enhance sensitivity of Gem, rather than SAHA and ps341, in KBGem clone.

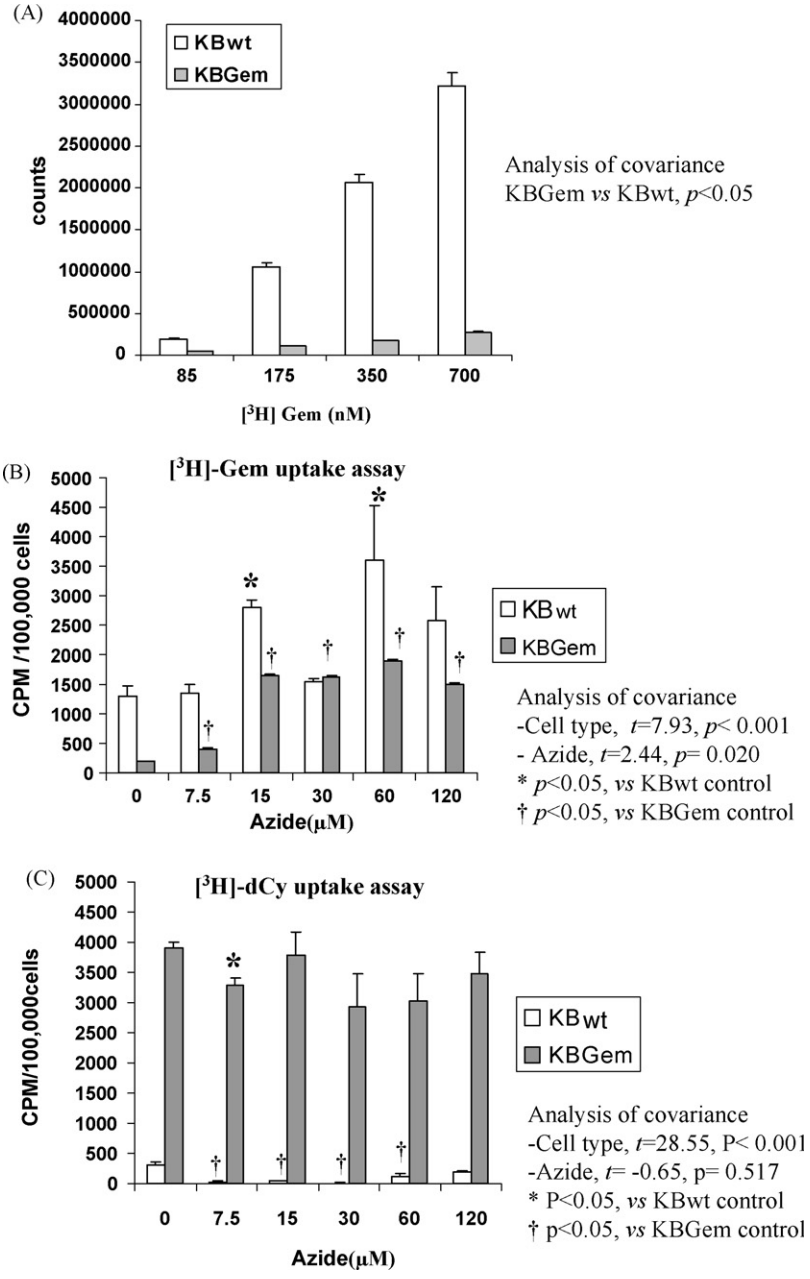

Fig. 4 – Determining [^{3}H]-Gem accumulation in KBGem under treatment of Azide. (A) [^{3}H]-Gem accumulation assay: 2×10^5 cells of KBwt and KBGem were plated onto 12-well plates, and [^{3}H]-Gem was than added at 85, 175, 350 and 700 nM. The cells were harvested after 72 h, and the whole cells were counted. Each done repeated in triplicate. (B) 2×10^5 cells were pre-treated with 0, 7.5, 15, 30, 60 and 120 μM of sodium azide for 3 h. Following the incubation of [^{3}H] labeled Gem (0.5 μCi/ml, 0.02 nmol/ml) for another 3 h, cells were washed with PBS twice and then harvested. One-fifth harvested cell pellet was counted using liquid scintillation counter. The DNAs were extracted from the remaining four-fifth of cell pellets. Results were adjusted with DNA concentration, and then normalized with corresponding control value. Analysis of covariance was conducted to test for statistic significance (t: t ratio; p: p value). (C) [^{3}H]-dCy uptake assay: The [^{3}H] labeled dCy (0.5 μCi/ml, 0.02 nmol/ml) was added into cell culture medium. The following procedure is the same as above.

The cytotoxicity of Gem (dFdCyd) occurs, mostly, through its incorporation into DNA as dFdCTP and causing masked chain termination [4,5]. Hence, the effect of azide on the rate of Gem incorporation into DNA was examined. Cells were treated with 0, 7.5, 15, 30, 60 and 120 μM of azide, the incorporation of [^{3}H] labeled Gem into DNA increased with increase of azide in KBGem and KBwt (Fig. 3B). It indicated sensitization to Gem by azide involves enhancing the rate of Gem incorporation into DNA. Taken together, the resistance to Gem in KBGem can be partially reversed by sodium azide.

3.6. Increase of [^{3}H]-Gem accumulation in KBGem by sodium azide

The uptake assay demonstrated that cellular accumulation of KBwt is almost 10-fold higher than in KBGem, when cells were incubated with 85, 175, 350 or 700 nM of [^{3}H]-Gem (Fig. 4A). When pre-treated with 0, 7.5, 15, 30, 60 or 120 μM of azide, the cellular accumulation of [^{3}H]-Gem increased in a dose (of azide)-dependent manner in KBwt and KBGem (Fig. 4B). Based on the covariance analysis, azide has a positive impact on [^{3}H]-Gem accumulation ($p < 0.05$); this effect on KBGem is much

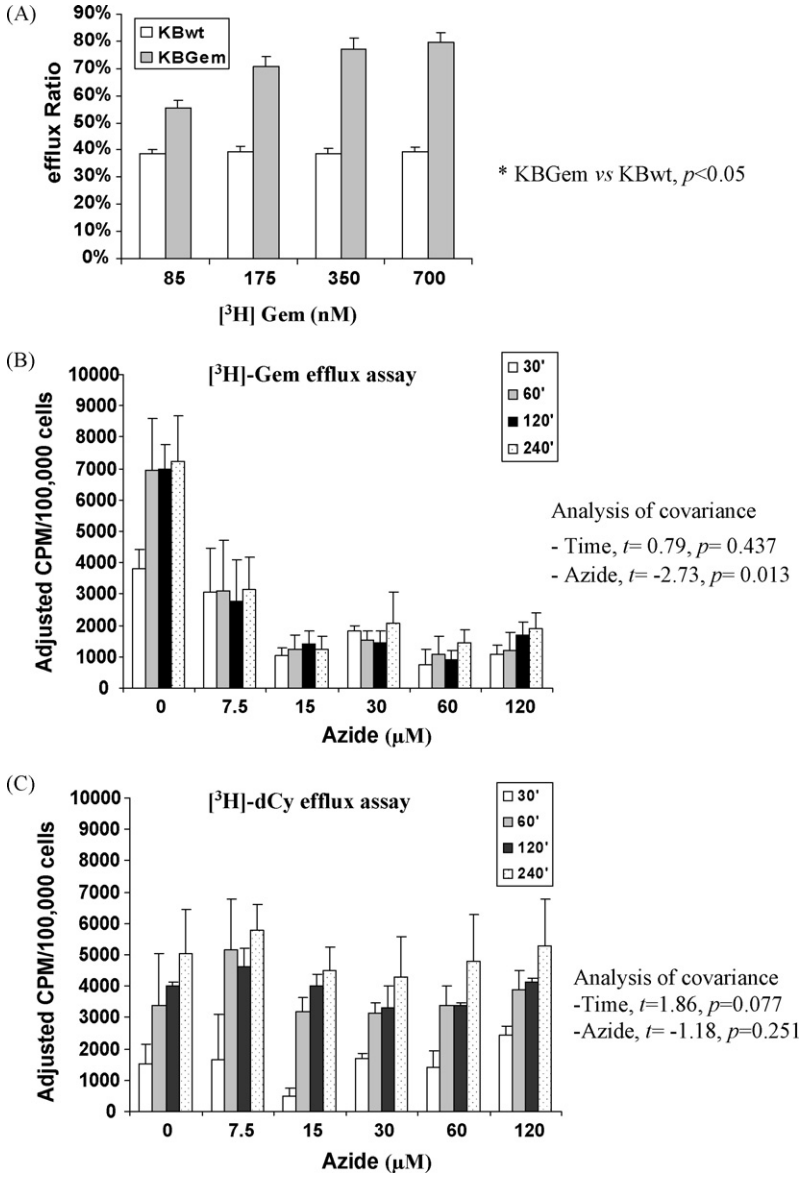

Fig. 5 – Suppression of [^{3}H]-Gem efflux by using azide. (A) [^{3}H]-Gem efflux assay: The cells were incubated with 85, 175, 350 and 700 nM of [^{3}H]-Gem for 24 h, washed and then changed with fresh medium for 1 h. Five hundred microliters from 2 ml medium was counted to evaluate [^{3}H]-Gem in efflux. (B) 2×10^5 KBwt cells were pre-treated with different dosages of sodium azide for 3 h. The cells were incubated with [^{3}H]-Gem (0.5 μCi/ml, 0.02 nmol/ml) for 3 h, then washed and re-suspended with medium containing 0.02 nmol/ml unlabeled Gem for 30, 60, 120 and 240 min. One hundred microliters from 1 ml medium was counted; and results were normalized by corresponding controls. The statistical significance was analyzed by using covariance analysis. (C) [^{3}H]-dCy efflux assay: The [^{3}H] labeled dCy (0.5 μCi/ml, 0.02 nmol/ml) was added into cell culture medium of KBGem. The following procedure is the same as above.

more pronounced than that on KBwt. The [^{3}H]-dCy uptake by KBwt is only 1/10th of that in KBGem; and sodium azide did not affect the accumulation of [^{3}H]-dCy in both KBwt and KBGem clones (Fig. 4C). Thus, sodium azide effectively enhances the accumulation of Gem, but not dCy, in KBGem.

As shown in Fig. 5A, the efflux assay showed that 38–39% of total uptake [^{3}H]-Gem could be detected in the medium after treated KBwt was freshly cultured with normal medium for 1 h. KBGem could efflux 60–80% of total uptake [^{3}H]-Gem in 1 h. The potential for Gem efflux increased in a dose-dependent manner. After incubating with fresh medium for 24-h, only 0.25–0.47% and 0.04–0.13% of absorbed [^{3}H]-Gem could be detected in KBwt and KBGem cells, respectively. As shown in Fig. 5B, under treatment of 7.5, 15, 30, 60 and 120 μM of azide in KBwt, the efflux of [^{3}H]-Gem (1 h) was reduced to 44.7%, 18.1%, 21.9%, 15.3% and 17.1%, respectively. Covariance analysis indicated that azide reduces efflux of [^{3}H]-Gem significantly ($p < 0.05$). However, the efflux of [^{3}H]-dCy did not change significantly when treated with different doses of azide ($p > 0.05$) (Fig. 5C). The above findings suggested that azide decreases [^{3}H]-Gem efflux, which cause increase of [^{3}H]-Gem accumulation.

4. Discussion

Drug resistance limits the application of Gem therapy in clinics treatments [6]. Further understanding of the mechanism underlying Gem resistance may help to enhance the efficacy of nucleoside analogs in cancer chemotherapy. An increase of hRRM2 has been causally linked to Gem resistance by our laboratory and others [15,24,28,29]. Besides hRRM2, the subunit hRRM1 and p53R2 have also been implicated in Gem resistance [14]. In addition, an increase of RR activity may contribute to resistance to fludarabine, a distinct nucleoside analogue, in human T-cells [30]. Gem sensitivity could be enhanced by inhibiting hRRM2 expression using siRNA, antisense RNA or RR inhibitor [29,31]. However, other investigations showed that RR inhibition can not completely reverse the resistance to Gem. Thus, an increase of hRR only partially accounts for the high-degree of resistance exhibited by several resistant clones.

Alteration of CCOX subunits expression has been observed in doxorubicin and adriamycin multi-drug resistant K562 cells [22,23]. Cells associated with resistance to chemotherapeutic agents and radiation characteristically have a lower mitochondrial membrane potential, smaller proton gradients in the mitochondria, a higher use of fat, etc. [32]. It indicated that modification of nuclear and/or mitochondrial genome(s) may occur following the step-wise selection for drug resistance in cancer cells. Nevertheless, the molecular mechanism of resistance to nucleoside analogues may differ from the resistance to DNA damaging drugs. In this study, four of nine gene fragments overexpressed in KBGem from RFDD-PCR analysis were identified as CCOX subunits. Further cDNA microarray analysis showed the ATP synthase (complex V) subunits are also increased in KBGem. The upregulation of CCOX subunit 1 and activity in KBGem have been confirmed via Western blot and enzymatic activity assay, respectively (Fig. 2). It led to an increase of free-ATP by almost four-fold in the KBGem (Fig. 2C). Due to its ability to selectively inhibit CCOX of the electron transport chain, azide has been widely used as an inhibitor to study CCOX and other hemoproteins [21]. Sodium azide could significantly enhance the [^{3}H]-Gem incorporation into DNA and improve Gem sensitivity by reducing Gem efflux in KBGem. The above data indicate that an increase of CCOX may contribute to Gem resistance in KBGem.

Several molecular signaling pathways may regulate how CCOX affects Gem resistance. Increasing CCOX may reduce the accumulation of Gem by modulating influx/efflux nucleoside transporter protein through free ATP. The four different types of transporter process for nucleoside analogues are summarized into: (1) equilibrative uniport, (2) substrate exchange, (3) concentrative Na^+- or H^+-dependent uptake and (4) substrate export through primary ATP-dependent active efflux pumps [33]. Nucleoside related transporters mainly include the equilibrative/concentrative nucleoside transporter (ENT/CNT), organic anion transporter (OAT) and organic cation transporter (OCT), peptide transporter (PEPT) and multi-drug resistance protein (MRP). In mammalian cells, at least two subtypes of equilibrative and three distinct Na^+-dependent concentrative nucleoside transporters are involved in nucleoside transport [34,35]. We found that the [^{3}H]-Gem accumulation in KBGem was only one-tenth of that to KBwt (Fig. 4A). After 1 h, almost 60–80% of [^{3}H]-Gem was effluxed in KBGem, which is significantly higher than that in KBwt (Fig. 5A). Another group reported that ATP-replete cells accumulated significantly less [^{3}H]-Gem than did ATP-depleted cells, which suggested an efflux mechanism for Gem [36]. Our observation also yielded similar results by using sodium azide, a CCOX inhibitor (Fig. 5B). Increasing the ATP concentration might enhance the efflux of Gem by affecting the Na^+/K^+ ATP pump channel. The most common ATP-dependent efflux pump for a cytotoxicity drug is the multi-drug resistance (MDR) transporter P-glycoprotein (P-gP) [37]. There is no evidence indicating that Gem is a substrate of the MDR system. However, the sensitivity of Gem was increased in a MDR P-gP overexpressing lung cell line [38]. Therefore, how CCOX regulates the Gem influx/efflux transporter still requires further investigation.

CCOX might synergistically interact with hRR and contribute to Gem resistance via increasing free ATP concentration. A recombinant mouse RR model demonstrated that free ATP regulates RR activity by binding to a specificity site (S-site), an adenine-specific site (A site) and a hexamerization site (H-site) of the RR large subunit [39–41]. An increase of free ATP drives RR subunits to form an active $(R1)_2(R2)_2$ dimer and a $(R1)_6(R2)_6$ hexamer [41]. Utilizing recombinant human RR subunits, an *in vitro* enzymatic activity assay showed that the holoenzyme hRRM1/hRRM2 and hRRM1/p53R2 have no activity without free ATP [42]. Several groups have demonstrated that overexpression of hRR subunits, including hRRM2, hRRM1 and p53R2, is associated with Gem resistance [14,15,29]. hRRM2 overexpression and hRR activity upregulation in KBGem was reported in several of our previous publications [15,24,28,31]. Therefore, increasing free ATP concentration may regulate hRR subunits to form excess active RR holoenzyme, which, in turn, causes an expansion in the dCTP pool.

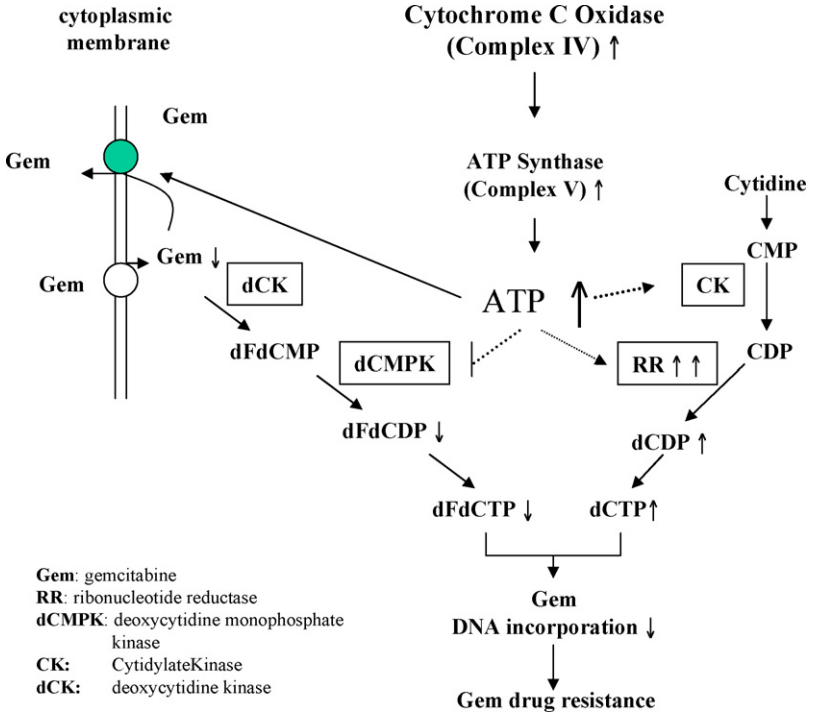

Fig. 6 – Increase of CCOX and disruption of Gem incorporation into DNA in KBGem. Gem is phosphorylated by dCK, dCMPK and NDPK, and then converted to the final formation, dFdCTP, to incorporate in DNA chain and cause DNA chain termination. Increase of CCOX activity and ATP synthase could inhibit dCMPK activity by increasing production of ATP, which reduces Gem metabolism into dFdCTP. Meanwhile, ATP activates the RR activity by binding to the activity site of hRRM1 subunit. Up-regulation of RR activity may expand the dCTP pool. Thus, synergetic effects of dCTP pool expansion and dFdCTP decrease may prevent Gem from incorporating into DNA and cause Gem resistance in KBGem.

Increase of CCOX activity may alter the Gem metabolic pathway via ATP in KBGem. The phosphorylation of Gem is mediated by dCK, dCMPK and NDPK enzyme. While cytidine is phosphorylated by uridine kinase and cytidylate kinase. This is followed by RR reduction, and phosphorylation by NDPK (Fig. 6). We previously showed that dCK dose not contribute to Gem resistant in KBGem [15]. Recently, an *in vitro* activity assay utilizing recombinant proteins demonstrated that free ATP inhibit the activity of dCMPK, but not cytidylate kinase (CK) [43]. When the ATP level increased from 2 to 5 mM, the efficiency of dFdCMP phosphorylation was reduced to about 64.7%. Whereas, ATP enhanced the CMP phosphorylation by mere 4.3% [43]. It suggested that the increase of CCOX activity results in an increase of ATP may reduce the phosphorylation of dFdMP in KBGem, and interruption of dFdCMP phosphorylation may block the dFdCTP formation. The decrease of dFdCTP and the expansion of the dCTP pool would prevent dFdCTP incorporation into DNA, which would contribute to Gem resistance in KBGem.

The above findings revealed that upregulation of free ATP level may represent a key molecular in Gem resistance signaling pathway in KBGem. The mechanisms of CCOX involving Gem resistance may occur through the following pathway (Fig. 6): (1) increasing CCOX enhances Gem efflux through increasing of free ATP; (2) reducing the dFdCTP formation through inhibiting dCMP kinase; (3) activating hRR activity and cytidylate kinase to provide excess dCTP to compete dFdCTP incorporation into DNA.

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