# Survivin May Enhance DNA Double-strand Break Repair Capability by Up-regulating Ku70 in Human KB Cells

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**Abstract.** *Background: Survivin, expressed in almost all types of human malignancies, functions as a key factor in radioresistance primarily by inhibiting apoptosis. This study was conducted to investigate whether survivin plays a role in the DNA repair process in the KB human squamous cell carcinoma cell line. Materials and Methods: A stable KB cell line overexpressing survivin was established through the use of pIRES2-EGFP vector containing the coding region of survivin. Cells were then irradiated with X-rays and evaluated for DNA double-strand breaks (DSBs) by comet assay and flow cytometry for phospho-histone γH2AX. The protein levels of some DSB repair genes were detected by Western blotting analysis. Results: Comet assay and flow cytometry for phospho-histone γH2AX showed that overexpression of survivin resulted in significantly fewer DSBs in irradiated cells. Among the DSB repair genes detected, the protein level of Ku70 was up-regulated in survivin-overexpressing KB cells. Conclusion: This finding suggests that survivin may enhance DSB repair capability in KB cells by up-regulating Ku70.*

Survivin, a member of the inhibitor of apoptosis (IAP) protein family, is expressed in embryonic tissue during fetal development, but either undetectable or expressed at a very low level in terminally differentiated adult tissues. However, it is overexpressed in almost all types of human malignancies (1-3). Survivin is known to play an important role in inhibiting radiation-induced apoptosis primarily *via* direct inhibition of caspase-related proteins, thereby promoting increased resistance to radiotherapy (4-6).

Chakravarti *et al*. found that down-regulation of survivin in the primary human glioma cell lines resulted in a significantly greater degree of double-strand breaks (DSBs) as determined by comet assay. In contrast, there appeared to be significantly fewer DSBs in cells overexpressing survivin (7). Likewise, Rödel *et al*. also reported such a phenomenon in colorectal cancer cells. They found that induction of phospho-histone γH2AX, a marker of recognized DSBs, increased significantly after irradiation in survivin short interfering RNA-treated cells (8).

It seems that disrupting survivin disrupts DSB repair capabilities of these tumor cells, *i.e*. survivin plays a role in the DNA repair process. However, it should be noted that DSBs may arise not only directly from irradiation, but also from irradiation-induced apoptosis (9, 10). However, no related discussions were given in the studies above. Therefore, further study is warranted to elucidate the role of survivin in the DNA repair process.

The present study was conducted to determine whether survivin regulates DSB repair in the KB human squamous cell carcinoma cell line.

## Materials and Methods

*Cell lines and cell culture*. The KB human squamous cell carcinoma cell line was selected for this study. KB cells were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Gaithersburg, MD, USA) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) and penicillin-streptomycin (100 U/ml) at 37˚C in a humidified incubator containing 5% $CO_2$.

*Establishment of a stable survivin-overexpressing KB cell line*. The full-length coding region of survivin was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using primers

*Abbreviations:* DSBs, DNA double-strand breaks; IAP, inhibitor of apoptosis; DMEM, Dulbecco's modified Eagle's medium; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TBS, tris-buffered saline; PBS, phosphate-buffered solution; HR, homologous recombination; NHEJ, nonhomologous end-joining.

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*Key Words:* Survivin, DNA double-strand breaks, DNA repair, radioresistance, squamous cell carcinoma.

(forward primer 5'-GGTACC GAA TCG CGG GAC CCG TTG-3'; reverse primer 5'-GGATCC TGG GAC CAG GCA GCT CCG-3') with internal Kpn I/BamH I sites (underlined). After being cloned by vector pGEM-T (Promega, Madison, WI, USA), the survivin cDNA was inserted into the Kpn I/BamH I site of pIRES2-EGFP (Clontech, Palo Alto, CA, USA). KB cells were transfected with the constructed vector pIRES2-EGFP-survivin or pIRES2-EGFP using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Stable plasmid-transfected clones were selected using G418 for 2 weeks. KB cells transfected with pIRES2-EGFP-survivin were named $KB_{survivin}$ cells. KB Cells transfected with pIRES2-EGFP were named $KB_{CON}$ cells.

*Western blotting*. Cells were lysed with denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer using standard methods. Protein lysates were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with tris-buffered saline (TBS) containing 0.1% Triton® X-100 and 5% nonfat milk overnight at 4˚C. For examination of survivin expression, the membranes were incubated with anti-survivin antibody (1:200; Santa Cruz Biotech, Santa Cruz, CA, USA), and anti-GAPDH antibody (dilution 1:5,000; Sigma-Aldrich) at 4˚C overnight; for examination of DSB repair gene expressions, the membranes were incubated with anti-DNA protein kinase (DNA-PKcs), -Ku70, -Ku80, -DNA ligase IV, -RAD50, -RAD51, -RAD52, -XRCC2, or -XRCC3 antibody (Santa Cruz Biotech). After washing, the membranes were incubated with HRP-conjugated mouse Ig at room temperature for 1 hour. Signal detection was carried out with an ECL system (Amersham Biosciences, Piscataway, NJ, USA).

*X-Irradiation*. Cells were irradiated with 2 Gy of X-rays. X-Irradiation was carried out at room temperature using an MBR-1520A-TW device (20 mA, 150 kV; Hitachi Medical, Tokyo, Japan) at a dose rate of 2.089 Gy/min.

*Flow cytometric analysis of apoptosis*. To determine whether irradiation-induced apoptosis occurred, apoptosis rates were determined at various time points (0 to 3 hours) after irradiation. Briefly, cells were collected and fixed with ice-cold 70% ethanol. Fixed samples were centrifuged at 250 ×g for 5 minutes, treated with RNase (0.25 mg/ml) and resuspended in propidium iodide (50 μg/ml) at room temperature. Propidium iodide-stained cells were analyzed by flow cytometry in a FACScan (Becton Dickinson, San Jose, CA, USA).

*Caspase-3 activity assay*. To further detect apoptosis, caspase-3 activity was analyzed using CaspACETM Assay System (Promega). In brief, cells were collected at various time points (0 to 3 hours) after radiation exposure, washed with phosphate buffer solution (PBS) twice and incubated in cell lysis buffer on ice for 15 minutes, then the supernatant fraction was collected by centrifugation. Assay was performed in triplicates in a 96-well plate based on the manufacturer's protocol. Caspase-3 activity was measured by colorimetry.

*Comet assay*. Cells were evaluated for DSBs at various time points (0 to 100 minutes) after radiation exposure using the comet assay according to the method described by Singh *et al*. (11). Briefly, single cells embedded in agarose were lysed in neutral lysis solution to remove proteins and were then electrophoresed. Staining was performed with ethidium bromide, with quantitative image analysis of tail length, which is associated with the degree of DSBs. One hundred cells for each experimental point were blind scored from two slides.

*Flow cytometry for phospho-histone γH2AX*. For the analysis of phosphorylated histone γH2AX, cells were collected at various time points (0 to 100 minutes) after radiation exposure and then analyzed by flow cytometry according to the method described by Rödel *et al*. (8). Briefly, irradiated cells were detached by accutase treatment for 15 minutes, pelleted by centrifugation and fixed in 70% ethanol for 2 hours at −20˚C. Before antibody labeling, samples were rehydrated with TBS (pH 7.4), centrifuged and incubated in tris-buffer with 0.1% Triton® X-100 for 10 minutes on ice. Cells were then incubated either with an FITC-conjugated anti-γH2AX-antibody (Upstate, Lake Placid, NY, USA) at a 1:250 dilution or an isotype control antibody for 2 hours in the dark. Cells were fixed in 1% paraformaldehyde in PBS and analyzed using a Coulter EPICS XL flow cytometer (Beckman Coulter, Hialeah, FL, USA). The mean fluorescence of the isotype-control was subtracted to eliminate nonspecific background staining for every sample.

*Statistical evaluation*. Experimental data are presented as mean±standard deviation (SD) from three or more independent experiments. Levels of significance for these data were calculated using Student's *t*-test. The level of significance was 0.05 (two-sided) in all statistical testing.

## Results

*Transfection with pIRES2-EGFP-survivin up-regulated survivin expression in KB cells*. To up-regulate survivin expression, pIRES2-EGFP-survivin was used to transfect KB cells. After 2 weeks, stable plasmid-transfected clones were selected using G418 (Figure 1). Western blotting analysis was performed to examine whether pIRES2-EGFP-survivin transfection altered the protein level of survivin. The results showed that survivin was markedly increased by 29.8±6.7% in KB cells transfected with pIRES2-EGFP-survivin when compared with KB cells. In contrast, no obvious change of survivin expression was observed in KB cells transfected with pIRES2-EGFP (Figure 2).

*Analysis of apoptosis in $KB_{survivin}$ and $KB_{CON}$ cells*. Experiments were carried out at various time points (0 to 3 hours) after radiation exposure to determine whether apoptosis was induced in $KB_{survivin}$ and $KB_{CON}$ cells. The results of flow cytometric analysis are summarized in Figure 3. It was shown that apoptosis rates after X-irradiation did not significantly change with time in either cell line ($p>0.05$), nor was there any significant difference between the apoptosis rates of $KB_{survivin}$ and $KB_{CON}$ cells at any time point ($p>0.05$). Caspase-3 activity assay was further performed to assess apoptosis. As shown in Figure 4, the results were consistent with those of the flow cytometric analysis.

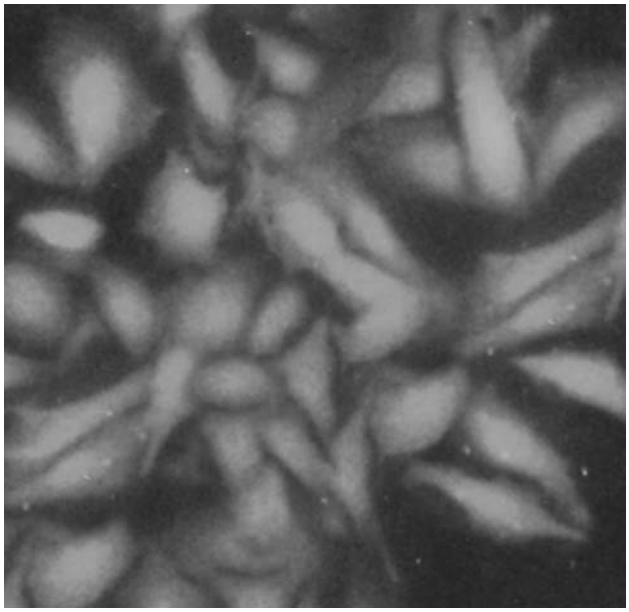

Figure 1. *Stable KB cell clone transfected with pIRES2-EGFP containing the coding region of survivin. pIRES2-EGFP-survivin was used to transfect KB cells. After 2 weeks, stable plasmid transfected clones were selected using G418.*

*Overexpression of survivin attenuated irradiation-induced DSBs as determined by comet assay*. Comet assay was performed under neutral elution conditions to determine DSBs at various time points (0 to 100 minutes) after X-irradiation. The tail length of migrating DNA is normally indicative of the degree of DSB under such condition. As shown in Figure 5, the comet tail length of $KB_{survivin}$ and $KB_{CON}$ cells at 0 minutes was 8.29±1.98 μm and 8.55±2.22 μm, respectively ($p>0.05$). At 40 minutes after X-irradiation, the comet tail length of $KB_{survivin}$ and $KB_{CON}$ cells reached a peak and was 18.00±3.01 μm and 26.02±3.89 μm, respectively. There was a significant difference between these lengths ($p<0.05$). The comet tail length of $KB_{CON}$ cells remained significantly elevated as compared with $KB_{survivin}$ cells at 60, 80 and 100 minutes ($p<0.05$). It can be seen that there was a significantly lower degree of DSBs in $KB_{survivin}$ cells compared with $KB_{CON}$ cells after irradiation.

*Overexpression of survivin resulted in decreased phospho-histone γH2AX induced by X-irradiation*. To further evaluate the degree of DSB, γH2AX was analyzed by flow cytometry after irradiation. As displayed in Figure 6, the percentage of γH2AX fluorescence of $KB_{survivin}$ and $KB_{CON}$ cells at 0 minute was 2.5±0.7% and 2.6±0.5% , respectively ($p>0.05$). At 60 minutes, the percentage of γH2AX fluorescence of $KB_{survivin}$ and $KB_{CON}$ cells reached a peak and was

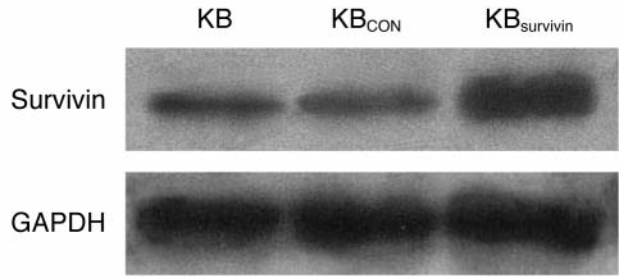


Figure 2. *Western blotting showed a significantly increased level of survivin protein in $KB_{survivin}$ cells compared with KB and $KB_{CON}$ cells. GAPDH was used as a loading control. Results shown are representative of three independent experiments.*

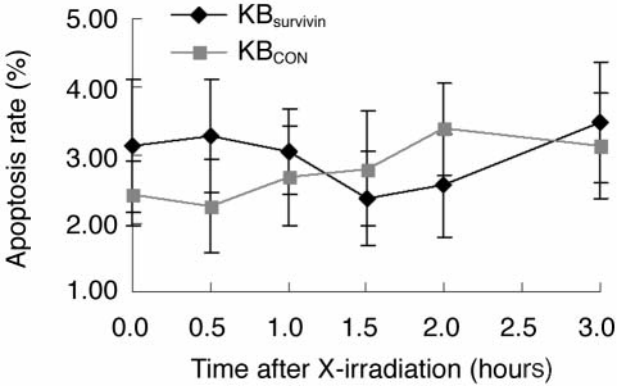


Figure 3. *Flow cytometric analysis of apoptosis. The results showed that apoptosis rates in each cell line did not change with time after X-irradiation and rates were not significantly different between $KB_{survivin}$ and $KB_{CON}$ cells ($p>0.05$).*

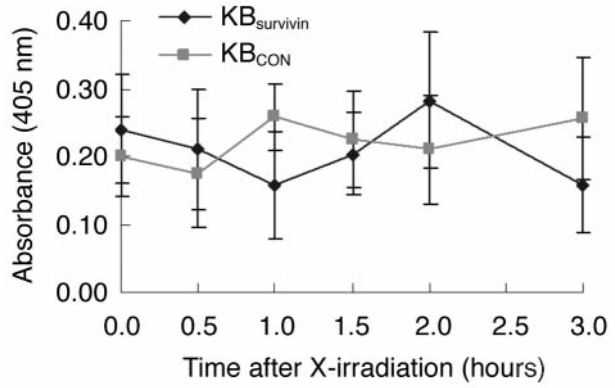


Figure 4. *Measurement of caspase-3 activity in irradiated cells. Cells were collected at various time points after X-irradiation and cell extracts were tested for caspase-3 activity by colorimetry. No significant difference of caspase-3 activity was found between various time points in each cell line nor between $KB_{survivin}$ and $KB_{CON}$ cells ($p>0.05$).*

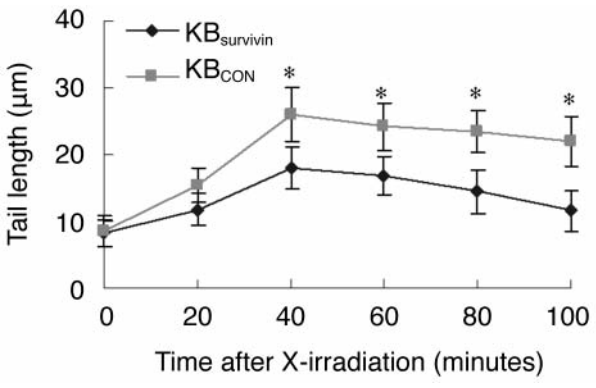


Figure 5. *Effects of overexpression of survivin on X-irradiation-induced double-strand breaks (DSBs) measured by comet assay. Comet assay was performed at various time points after irradiation. The results showed that overexpression of survivin in KB$_{survivin}$ cells resulted in a significantly fewer DSBs following irradiation as compared with KB$_{CON}$ cells. *p<0.05 (compared with KB$_{CON}$ cells).*

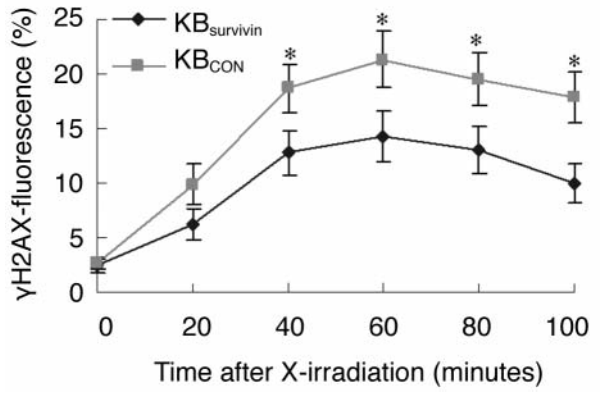


Figure 6. *Flow cytometric analysis of phospho-histone γH2AX after irradiation. The results showed that the population of γH2AX-positive KB$_{survivin}$ cells was less than that of KB$_{CON}$ cells, suggesting that there were a significantly fewer X-irradiation-induced DSBs in KB$_{survivin}$ cells. *p<0.05 (compared with KB$_{CON}$ cells).*

14.3±2.3% and 21.3±2.6%, respectively ($p$<0.05). The percentage of γH2AX fluorescence of KB$_{CON}$ cells remained significantly elevated as compared with that of KB$_{survivin}$ cells at 60, 80 and 100 minutes ($p$<0.05). The results showed that the population of γH2AX-positive KB$_{survivin}$ cells was significantly less than that of KB$_{CON}$ cells after X-irradiation, indicating that there was a significantly lower degree of DSB in KB$_{survivin}$ cells as compared with KB$_{CON}$ cells.

*Western blotting analysis for DNA double-strand break repair genes*. To further elucidate the possible underlying mechanisms by which survivin enhances DSB repair capability, Western blotting was carried out to detect the protein level of some DSB repair genes (*DNA-PKcs, Ku70, Ku80, DNA ligase IV, RAD50, RAD51, RAD52, XRCC2* and *XRCC3*). The results showed that *Ku70* was up-regulated in KB$_{survivin}$ cells (Figure 7). Densitometric analysis yielded a 19.5±4.3% increase of Ku70 protein expression in KB$_{survivin}$ cells as compared with KB$_{CON}$ cells ($p$<0.05). Except for *Ku70*, no obvious changes were found in the protein level of other DSB repair genes we examined (data not shown).

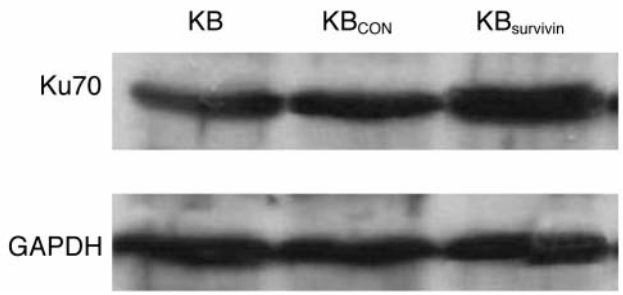


Figure 7. *Western blotting showed a statistically significant increase of Ku70 protein in KB$_{survivin}$ cells as compared with KB and KB$_{CON}$ cells (p<0.05). GAPDH was used as a loading control. Data represent one out of three separate experiments.*

## Discussion

In recent years, survivin has been highlighted in many studies, which is largely attributed to its unique structure, expression, regulation and function (12-14), and it has emerged as a promising therapeutic target in cancer therapy (15-17). Previous studies have established that survivin is an important mediator in carcinogenesis, acting as a radioresistance factor mainly by inhibiting caspase activity. However, the actions of survivin in irradiated cells are not fully understood. There seems to be additional mechanisms by which survivin enhances tumor cell survival upon radiation exposure. It was found that survivin can regulate tumor cell metabolism and the cell cycle, thereby suppressing radiation-induced cell death.

More importantly, survivin was suggested to play a role in the DSB repair process (7, 8). As radiation has been demonstrated to significantly improve clinical outcomes of patients with many types of tumor, it is quite possible that elucidating this novel function of survivin may help to overcome radioresistance and improve outcome for these patients. This study was conducted to investigate whether survivin can enhance DSB repair capability in KB cells. We firstly transfected KB cells with a plasmid containing the full-length coding region of survivin and established a stable cell line which overexpressed survivin, as confirmed by Western blotting.

As discussed above, DSBs can be generated during apoptosis. If apoptosis took place after irradiation, DSBs detected by comet assay or any other analysis may not be primarily attributed to irradiation directly. So above all, the possibility that DSBs measured in this study result from apoptosis had to be excluded. To investigate whether the DSBs measured involved apoptosis-related events due to irradiation, apoptosis was firstly analyzed. The results of the apoptosis assays showed that there was no indication of radiation-induced apoptosis in either $KB_{survivin}$ or $KB_{CON}$ cells within 3 hours after X-irradiation. Furthermore, DSBs were observed 100 minutes after irradiation in this study, which is too short a time for the induction of apoptosis-driven DNA degradation. This suggested that DSB measured either by comet assay or by analysis of γH2AX in this study were unlikely to have involved apoptosis-related events resulting from radiation exposure. Hence in our opinion, the degree of DSBs measured in the present study reflected the DSBs directly caused by X-irradiation.

We then detected DSBs by comet assay. The results showed that overexpression of survivin led to a lower degree of DSBs. To further confirm this phenomenon, flow cytometry for γH2AX was carried out and the results were in accordance with those of the comet assay. Our finding indicated that overexpression of survivin enhanced the DSB repair capability in KB cells.

In mammalian cells, DSBs may be rectified by both homologous recombination (HR) and nonhomologous end-joining (NHEJ) (18, 19). The HR system involves a large number of proteins, including the core homologous recombination proteins RAD51, the RAD51 paralogs (XRCC2, XRCC3, RAD51B, RAD51C, RAD51D), RAD52, RAD54, RAD54B, the MRE11-RAD50-NBS1 (MRN) complex and the BRCA proteins (20–22). In contrast to homologous recombination, the NHEJ system uses little or no homology at all to repair the break. The main components of the NHEJ system in eukaryotes are the catalytic subunit of DNA-PK, which is recruited by Ku protein, a heterodimer of Ku70 and Ku80, as well as XRCC4 protein and DNA ligase IV. The nuclease complex RAD50/MRE11/XRS2 can be also involved in this process (23–25).

For this reason, we further detected the protein levels of some of these DSB repair genes to elucidate the possible underlying mechanisms by which survivin enhances DSB repair capability. Our results showed that *Ku70* was demonstrably up-regulated by 19.5±4.3% in survivin overexpressing KB cells. As one of three subunits of DNA-PK, Ku70 and Ku80 proteins form Ku heterodimers which with a catalytic subunit DNA-PKcs form DNA-binding regulatory subunits of DNA-PK. The Ku protein-initiated process of DSB repair occurs by activating DNA-PKcs after binding to DSB. In addition, Ku70 has a number of additional activities, including nonspecific DNA end-binding activity (26), ATPase and DNA helicase activities (27), sequence-specific DNA-binding activities (28), and regulating the precise structure of telomeric termini, possibly *via* controlling the access of nucleases and recombinases (29, 30).

Except for *Ku70*, no obvious changes were found in the protein levels of other DSB repair genes we examined. It should be noted that we only detected some of DSB repair genes and there are probably other as yet unidentified proteins involved in the HR and NHEJ systems. Hence, we cannot exclude the possibility that other DSB repair genes may be regulated by survivin.

In summary, our results suggested that overexpression of survivin might enhance DSB repair capability in KB cells by up-regulating *Ku70*. However, the exact role of survivin in DSBs and how to link survivin to *Ku70* up-regulation need to be further studied to help overcome radioresistance in tumors.

## Acknowledgements

This work was supported by the Basic Research Program (2006XZ10 and 2008XG8) and Doctoral Start-up Foundation of Wuhan University of Science and Technology.

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*Received June 7, 2008*
*Revised September 22, 2008*
*Accepted November 6, 2008*