Genetic alterations after carbon ion irradiation in human lung adenocarcinoma cells

EMMANOUIL FOKAS*, AN YOU*, JANKO JURICKO, RITA ENGENHART-CABILLIC and HAN-XIANG AN

Department of Radiotherapy and Radiation Oncology, Philipps University Marburg, Baldingerstrasse, 35043 Marburg, Germany

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Abstract. The aim of this study was to investigate the difference in gene expression profiles of human lung adenocarcinoma cells and identify genes whose expression is altered by heavy ions but not X-rays. The lung adenocarcinoma cell line A549 was irradiated with carbon ion beams and X-rays using biologically equivalent doses (2 Gy and 6 Gy, respectively). The transcriptional profiling was determined with a high density cDNA microarray containing 11.800 genes, and genetic network and gene ontology analysis was performed. The changes in selected genes involved were validated by quantitative real-time polymerase chain reaction (qRT-PCR). The microarray analysis identified 49 mapped, network-eligible genes, the expression level of which was altered by carbon ions but not by X-rays. From these, 29 were upregulated while 20 genes were downregulated 4 h post-irradiation with carbon ions in A549 cells, as compared to the control. Among these, three genes (CCND2, RARG and E2F5) were involved in the aryl hydrocarbon receptor signalling and G1/S cell cycle checkpoint pathways. The microarray data were corroborated by qRT-PCR analysis of the selected genes ($p<0.05$). Our findings provide information on the genetic signature of carbon ions in human lung adenocarcinoma cells and add to the understanding of the complicated molecular response to carbon ion irradiation.

Introduction

Lung cancer is the most common malignancy and the major cause of cancer-related deaths of men in industrialized countries (1). Conventional radiotherapy consists an effective modality for several human cancers (2,3). However, a dose escalation is often imperative to improve therapeutic outcome, with the cost of increased toxicity (4).

The superior biophysical and biological profiles of particle beams such as carbon beam and protons with excellent dose localization and sparing of normal tissues makes them highly attractive for treating malignant tumors including lung cancer, whereas more research is currently warranted to better evaluate the cost-effectiveness of particle therapy (5-8). The phase I and II clinical trials on carbon ion radiotherapy for stage I non-small cell lung cancer (NSCLC) have showed promising results regarding local control and overall survival (5,6). A recent meta-analysis, though, concluded that survival rates for particle therapy were higher than those for conventional radiotherapy, but similar to sterotactic body radiotherapy in stage I inoperable NSCLC, suggesting that particle therapy may be more beneficial in stage III NSCLC, especially in reducing adverse events (8).

Despite the advance in understanding the radiobiological aspects of carbon ions (C12), their molecular mechanisms remain largely unknown. cDNA microarray technology is an effective tool to analyze transcriptional genetic alterations in response radiation (9). Recent microarray studies demonstrated different expression pattern in squamous cell carcinoma-derived cells, melanomas and breast epithelial cell irradiated with C12 and X-rays (10-13).

We investigated the global expression changes of A549 human lung adenocarcinoma cell line after irradiation with C12 and X-rays by using high-density cDNA microarray containing 11.800 unique genes and transcripts. A comprehensive network and gene ontology analysis was performed using the Ingenuity Pathway Analysis tool (IPA) to identify networks of interacting genes and their corresponding canonical pathways. Our primary aim was to study the difference of gene expression between carbon ions and X-rays in human lung adenocarcinoma cells and identify genes which are differentially expressed by heavy ion irradiation and not substantially altered by X-rays.

Materials and methods

Cell line and culture condition. The human lung adenocarcinoma cell line A549 was obtained from American Type Culture Collection (Rockville, MD) and maintained in RPMI medium supplemented with 10% fetal calf serum and 1% penicillin, streptomycin (Sigma, Germany) at 37˚C in 5% CO_2.

Correspondence to: Dr Han-Xiang An, Department of Radiotherapy and Radiation Oncology, Philipps-University Marburg, Baldingerstrasse, 35043 Marburg, Germany
E-mail: an@med.uni-marburg.de

*Contributed equally

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Table I. Primers used for qRT-PCR of genes with altered expression after carbon ion irradiation.

Gene	Forward	Reverse	Size (bp)
RARG	TACCACTATGGGGTCAGC	CCGGTCATTTCGCACAGCT	195
CCND2	TGGAGCTGCTGTGCCACG	GTGGCCACCATTCTGCGC	181
E2F5	TCAGGCACCTTCTGGTACAC	GGGCTTAGATGAACTCGACTC	145
CDC14B	GTGCCATTGCAGTACATT	AGCAGGCTATCAGAGTG	123
GAPDH	CATCTCTGCCCCCTCTGCTGA	GGATGACCTTGCCCACAGCCT	305

Bp, base pair.

Table II. Merged genetic networks identified in A549 cells irradiated with carbon ions.

Network	Gene	Function	Score[a]
1	Calmodulin, CAMK1D, CASP8AP2, CCND2, CD70, FAS, DDB2, FAIM, FGF13, GAP43, HBEGF, IL31, Interferon α, Jnk, KIF11, LGALS7, MAPK, NCOA7, NFkB, NRIP2, NUAK2, P38 MAPK, PI3K, PKMYT1, PPM1D, PSMC3IP, RARG, RIPK4, RNA polymerase II, SH2B1, T3-TRß, THRB, TIMP3, TRIM32	Cell cycle, Hematological disease, Gastrointestinal disease	32
2	ARID1B, ß-estradiol, BTBD10, BUB1, C11ORF51, CDC25C, CDKN1A, CKS2, CKS1B, CRADD, DCTPP1, DHPS, E2F4, E2F5, EDN1, GHRHR, GTF2H4, KLK4, MIR292, MIR106A, MIRLET7B, MYC, NIF3L1, NPHP4, PCNA, PCTK3, PKMYT1, PLEKHG3, POLS, PSAP, TFDP3, TYMS, UBE2C, UNG, ZBED1	Cell cycle, Cell signaling, Connective tissue development and function	19
3	ABL1, APBA2, CDC42, CDC42BPA, CDC42BPB, CDC42EP1, CKS2, Cofilin, CTBS, EGF, ERBB, FLII, GRB2, HIST1H1B, HNRNPR, HRAS, hydrogen peroxide, IL5RA, LGALS7, LIMK2, MAPKAP1, MYC, NCKIPSD, OAZ2, PHKA2, PLK3, PVR, RCC1, RELA, RPL26, RPL21, RPL7A, SNRPG, Timp, UBE2C	Cell cycle, Cell death	17
4	B3GAT3, BRE, CD70, CDC14B, CTSD, FAM179B, FAM40A, FGFR1OP2, HIC2, HTT, KCNH2, MIRN326, PDCD10, PDK2, PLK3, PPHLN1, PPL, PPME1, PPP1R3C, PPP2R1A, PPP2R2A, RP6-213H19.1, SFXN3, SIK1, SIKE1, STK24, STK25, STRN, STRN3, TAX1BP1, THRSP, TNF, TP53, TRAF3IP3, UBQLN2	Cell death, Amino acid metabolism, Molecular transport	19

Network-eligible, overlapping genes (n=43) whose expression was modified after carbon ion irradiation, but not by X-rays have been underlined. The rest of the genes either did not show any significant change or were not detected from the array; [a]A score >3 was significant.

Irradiation using X-ray and C12 beams. A549 cells seeded on 35-mm Petri dishes (70-80% confluent) were irradiated with X-rays (250 kV, 16 mA, dose range 0 to 12 Gy) or carbon ion (C12; 9.8 MeV/nucleon on target, LET 170 keV/μm, dose range 0 to 6 Gy) at the UNILAC facility at GSI. Details of C12 ions irradiation have been previously described (14).

RNA extraction and microarray hybridization. Total RNA from unirradiated and irradiated cells was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. RNA was treated with 30 U DNase, and purified again (RNeasy kit, Qiagen). The quality of RNA was determined by electrophoresis on a denaturing agarose gel. The cDNA labeling with Cy5- and Cy3-dyes and microarray hybridizations were performed as previously described (15). The chips used in this study contained 12,000 clones from the human sequence-verified UniGene cDNA sets gf200, gf201 and gf202 (ResGen). Each cDNA was spotted twice per chip. Cells at 4 h after IR were selected as treated sample and compared each other as well as with unirradiated cells. In order to balance fluorescence intensities for the two dyes, each experiment was performed as sandwich hybridization including reverse labeling with Cy5 and Cy3 dye for a second microarray. This provides a replicated

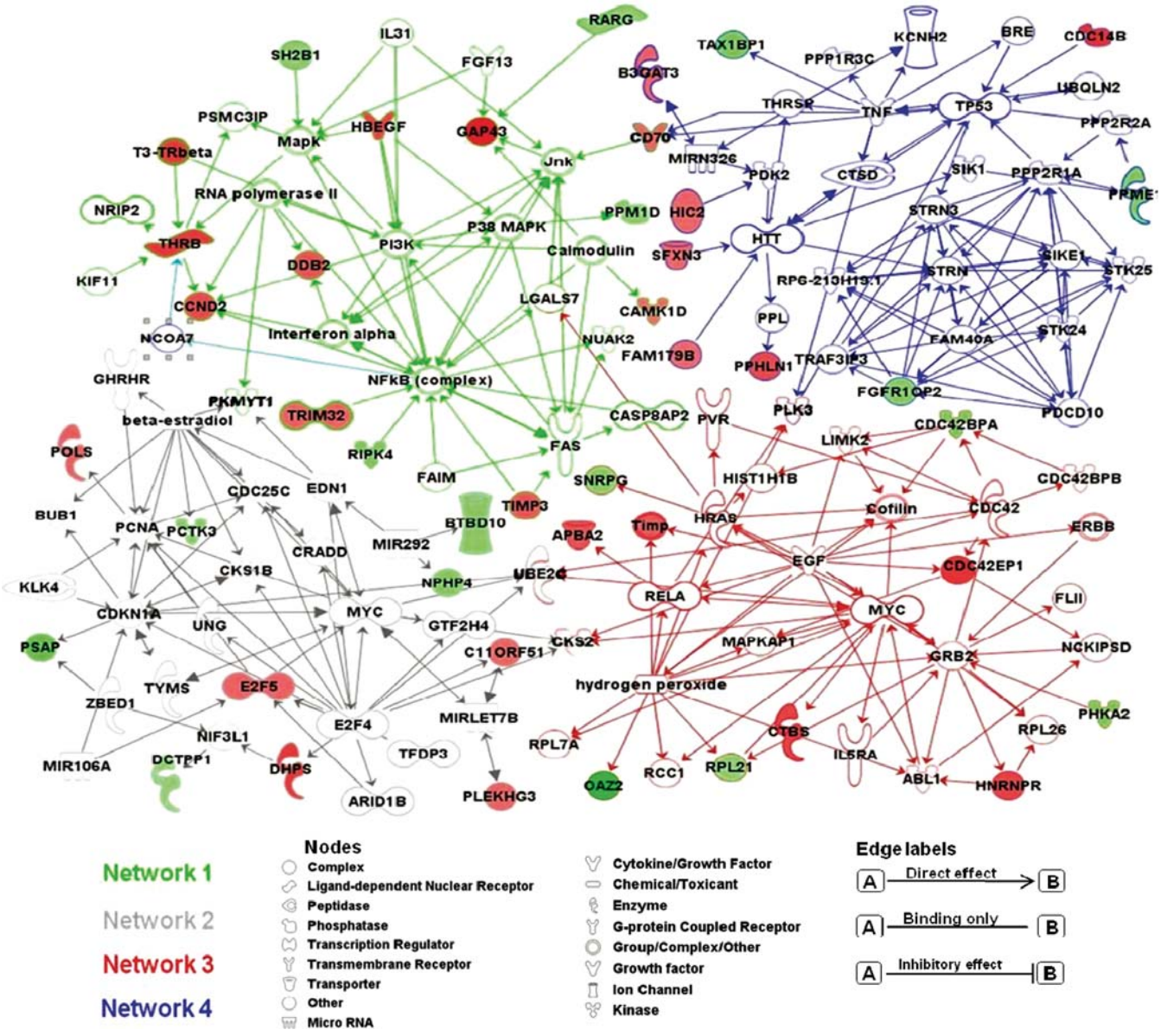


Figure 1. Interrelated networks of genes whose expression was modified after C12 irradiation. In total, four important networks of interrelated genes were identified. The four networks (green, network 1; orange, network 2; red, network 3; blue, network 4) were merged by overlapping genes (in bold). The degree of either upregulation (red) or downregulation (green) is reflected from the intensity of node colour.

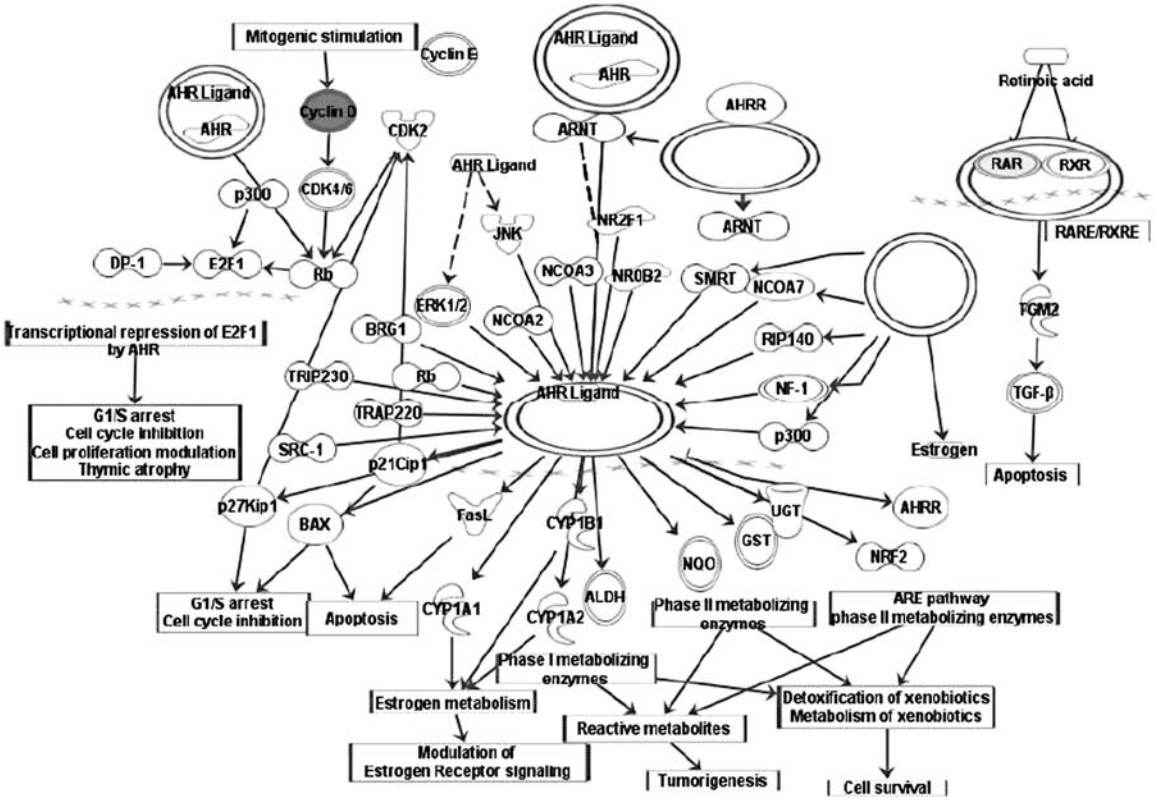
measurement for each hybridization, which can be used for quality control and for reduction of technical variability.

Network and gene ontology analyses. The fluorescent data from all experiments were normalized and analyzed for differentially expressed genes by using the Genesis package (16). Genetic network and functional classification of differentially expressed genes were investigated by using the IPA software (Ingenuity Systems, Mountain View, CA), a web-delivered tool that enables the discovery, visualization, and exploration of molecular interaction networks in gene expression data (17).

Quantitative real-time PCR. Validation of the expression of selected candidate genes was performed using SYBR Green Mixtures (Abgene, Darmstadt) on MyiQ thermocycler (Bio-Rad, Munich). Primer sequences used for candidate genes and glyseraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control are shown in Table I. Each reaction contained 12.5 μl of 2x iQ SYBR Green mixes, 2.5 μl of cDNA sample, and 70 nM gene-specific primers in a final volume of 25 μl. All reactions were performed in triplicates. The PCR reaction was evaluated by melting curve analysis and the calculations for determining the relative level of gene expression were made using the cycle threshold (Ct) method. The mean Ct values from triplicate measurements were used to calculate relative expression of the target genes with normalization to GAPDH used as internal control using the 2-$\Delta\Delta$Ct method.

Statistical analysis. The association between the transcriptional expression of the irradiated and unirradiated cells was analysed

A.



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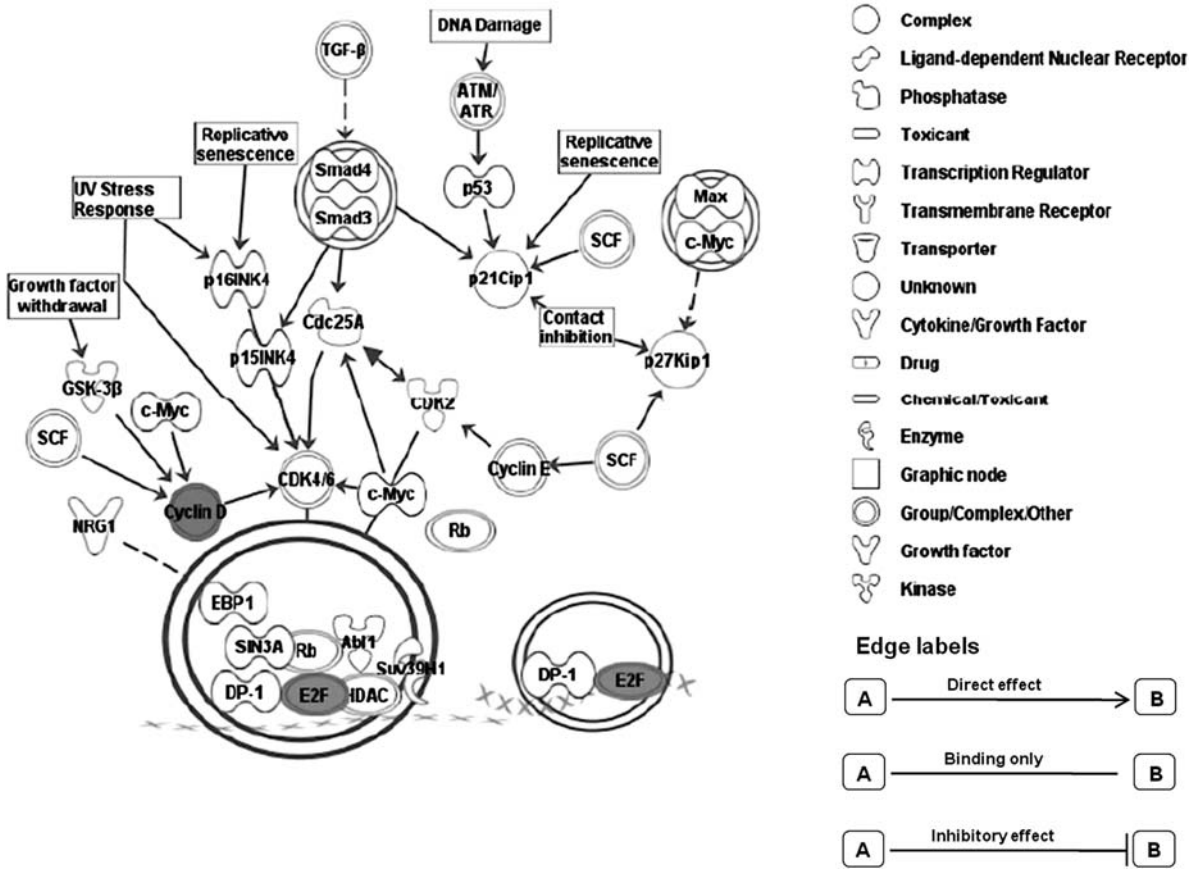

Figure 2. Important canonical pathways according to altered expression of genes after C12 irradiation. The AhR signaling (A) and the cell cycle: G1/S checkpoint regulation pathway (B) were on the top of the significant pathways list. Gray notes indicate upregulated genes. Genes which were not specified but integrated into the network through relationships are shown as white. For illustration purposes, the AhR pathway was simplified including only the genes with altered expression.

Table III. Canonical pathways in carbon ion-irradiated genes.

Ingenuity canonical pathways	P-value
Aryl hydrocarbon receptor signaling	0.007762
Cell cycle: G1/S checkpoint regulation	0.012589
p53 signaling	0.030903
Glioma signaling	0.033884
Pancreatic adenocarcinoma signaling	0.038019
Hereditary breast cancer signaling	0.048978
Lipid antigen presentation by CD1	0.049234

Table IV. Irradiation-induced fold changes in selected genes after carbon ions and X-ray treatment.

Gene sybol	Gene name	Fold changes	
		Carbon	X-ray
CCND2	Cyclin D2	2.35	1.26
RARG	Retinoic acid receptor-γ	0.42	0.88
E2F5	E2F transcription factor F5	2.29	1.24
CDC14B	CDC14B protein phosphatase	2.84	1.43

The differential expression in genes irradiated with carbon ions and X-rays is depicted.

using the Students t-test. The Fisher's test was used to analyze the significance of the canonical pathways and genetic networks identified by the IPA tool. A $p<0.05$ was considered significant.

Results

Identification of different expressed genes after X-ray and C12 irradiation. C12 have an increased RBE and previous reported RBE value of A549 cells was approximately 2.93 irradiated with highly energy carbon ions (18), we therefore used 1/3 the physical doses of X-ray for doses of C12 beams to compare the expression profile of A549 cells at the biologically effective doses (BED).

We performed profiled gene expression and compared expression in cells at 4 h after C12 (2 Gy) and X-ray irradiation (6 Gy) by examining 11,800 transcripts. We aimed to identify genes which may be affected in the response to C12 ions, but not after X-ray irradiation in lung cancer cells. Micro-array analysis revealed a significant alteration in the expression of 49 genes (at least 2-fold) after C12 irradiation and not altered by X-rays, as compared with control unirradiated cells. Of these 49 differentially expressed genes identified, 29 and 20 genes were up- and down-regulated, respectively.

Network and gene ontology analyses. The genetic network and functional classification of the differentially expressed genes were executed using the IPA tool. In total, all of the 49 genes were mapped, network-eligible and classified into genetic networks. From the 49 genes, 43 genes were part of networks that shared at least one overlapping gene in common and were merged together. In our study, 4 merged networks were identified (Fig. 1). The IPA tool depicted important biological pathways associated with these 4 merged networks (Table II). Different molecular events directly relevant to cancer were identified i.e. cell cycle, cancer and cell death signaling, cell signaling (Table II).

The rest of the 6 genes which were altered by C12, but not by X-rays, were detected in separate networks which, in contrast to the 4 overlapping networks mentioned above, were not overlapping between them due to the lack of commonly-shared gene. These included i) cellular development: HPS1 (up-regulated), ii) molecular transport and metastasis: ABCC5 (up-regulated), SYDE1 (down-regulated), TSPAN17 (down-regulated), iii) cell morphology: C90RF75 (down-regulated) and iv) immune response: RNF219 (up-regulated).

Gene ontology analysis detected canonical pathways with known implications in cancer (Table III). Among these, statistically significant pathways such as aryl hydrocarbon receptor (AhR) signaling ($p=0.0077$) and G1/S cell cycle checkpoint ($p=0.012$) were identified (Fig. 2A and B; Table III). From the genes detected, CCND2 was involved in both pathways. The others included RARG and E2F5 (Fig. 2A). Four of the genes with altered expression after C12 irradiation, in microarray analysis, are shown on Table IV.

Validation of microarray data by real-time PCR. To validate the results of microarray experiments, 4 differentially expressed genes involved in the AhR signaling and cell cycle were verified by qRT-PCR using RNA isolated at 4 h after IR. We analyzed the same RNA used for the microarray experiment. The expression levels of selected genes were similar with microarray data at 4 h post-irradiation with C12, as compared with the control and are shown in Fig. 3 ($p<0.05$). Only minor, non-significant changes were observed after X-ray irradiation, as compared with the unirradiated control group, validating our microarray data.

Discussion

Heavy ions such as C12 beams are characterized by higher RBE than X-rays and enhanced ionization density in the individual tracks of the heavy particles, where DNA damage becomes clustered and therefore more difficult to repair (7). In comparison to the low-LET proton and photon beams, carbons are of mixed LET with low LET in the entrance channel and high LET in the target volume and therefore DNA damage is restricted to the end of their range (19). Altogether, the superior biophysical and biological profile of carbons over conventional radiotherapy enables more precise dose localization for the therapy of anatomically complex and radioresistant malignancies, sparing the normal tissues from the severe side effects of conventional radiation (20).

Although a recent study demonstrated the ability of C12 to suppress the metastatic ability of lung cancer cells (21), to date, no detailed report on the gene expression profiles of lung

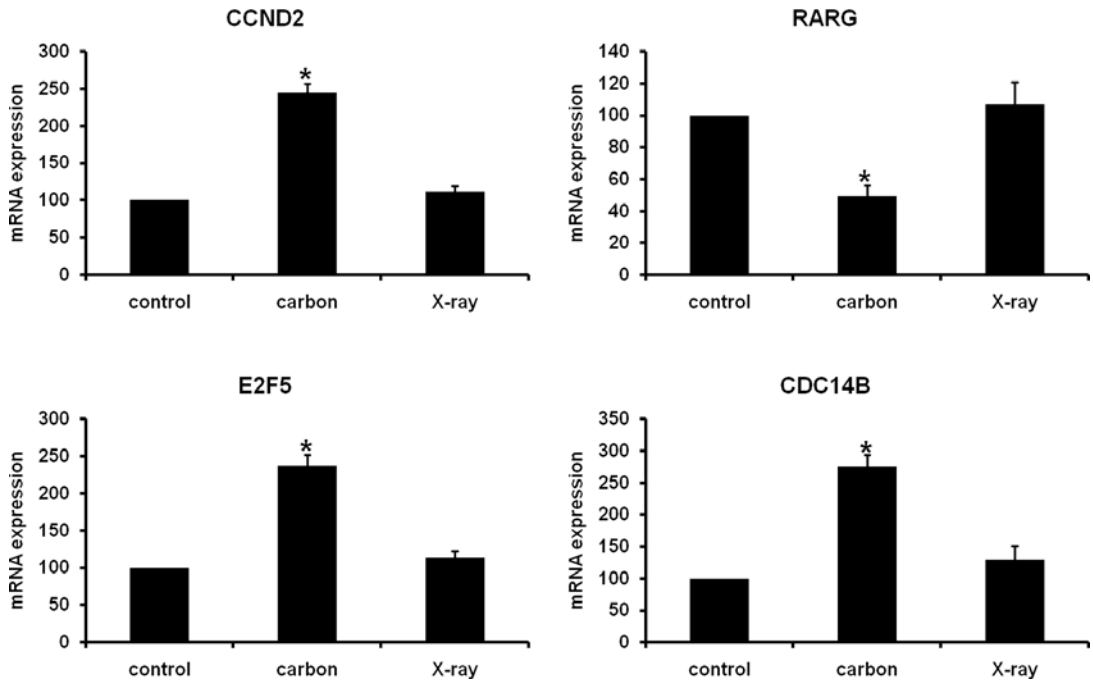

Figure 3. Validation of selected differently expressed genes in A549 cells at 4 h after carbon C12 and X-ray irradiation using qRT-PCR. The results of transcriptional expression were normalized to the values of GAPDH gene and then expressed as a percentage in comparison to the unirradiated, control cells (0 Gy). The significance in genetic expression changes after C12 was confirmed ($p<0.05$, Student's t-test). No major change was observed after treatment with X-rays. Data are expressed as mean ± SD.

cancer cells after C12 irradiation has been published. Our primary purpose was to detect genes specifically altered by C12 irradiation but not X-rays, which could account for the increased efficacy of heavy ions, as compared to conventional radiation. We irradiated cells with C12 ions and X-rays using BED. We employed a gene chip to analyze gene expression changes after C12 irradiation in A549 human lung adenocarcinoma cells. We identified 49 mapped genes, whereby 29 were overexpressed while 20 genes were downregulated 4 h post-irradiation with C12 and not altered by X-rays. Four major networks were detected and merged via overlapping genes. Network 1, 2 and 3 were closely associated with the cell cycle while network 3 and 4 included genes involved in the cell death machinery signaling.

The genetic mechanisms responsible for the increased effectiveness of heavy ions are currently under investigation but only few exist to date. Higo *et al* (10) performed a microarray study in oral squamous cells carcinoma cells (OSCCs) after C12 irradiation and focused specifically on the genes which showed no change in their expression after X-ray irradiation, similarly to our study. A series of 98 genes were modified upon C12 irradiation, among which SPHK1, a secondary messenger in cellular proliferation and survival. In a similar study from the same group, 84 genes were found to be modulated while the transforming growth factor ß-signaling pathway (TGF-ß) and cell cycle: G1/S checkpoint regulation pathway were those most affected from C12 in OSCCs (11). Matsumoto *et al* (12) detected transcriptional changes in several genes involved in the regulation of cell cycle and G2/M arrest. C12 induced upregulation of stress-responsive and cell-communication genes common to different tumor models *in vivo*, which could explain their efficacy (22).

Moreover, we investigated the canonical pathways in response to C12 irradiation. The study revealed that the AhR signaling and the cell cycle G1/S DNA damage checkpoint regulation were highly significant, detected on the top of the list. The AhR signaling has been previously implicated in the pathogenesis of lung cancer (23,24). One gene (CCND2) was upregulated and one (RARG) was downregulated in this pathway. The family of retinoic acid receptors (RAR) is dysregulated in lung cancer and repression of RAR-ß is often observed in lung carcinogenesis (25,26). Although RARG has been implied in tumor development (27), and retinoic acid-induced cell cycle arrest and apoptosis, its functions are not well characterized while its overexpression does not confer resistance to chemotherapy (28,29). The downregulation of RARG found in our study might have an impact on the regulation of cell cycle and apoptosis induced by carbon irradiation. More studies on the function of RARG will clarify this issue.

The importance of cell cycle in preservation of genomic stability and response to radiation-induced damage has been thoroughly investigated (30). Irradiation causes delays in the movement through the different phases of cell cycle while cells found in S- and plateau-phase are more radioresistant than cells in G2- or mitotic phase (30,31). Radioresistance to conventional radiotherapy can be overcome by C12 which can induce cell cycle arrest much more potently than low-LET beams (32,33). For increasing LET values, a general decrease of the variation in sensitivity, according to the phase, has been observed (34). In our study, CCND2 and E2F5 were the two genes associated with the cell cyle: G1/S checkpoint regulation. CCND2 forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition by interacting with the

tumor suppressor protein Rb. Upregulation of CCND2 can decrease proliferation and growth of tumors both *in vitro* and *in vivo* (35,36). Interestingly, overexpression of cyclin D2 protein efficiently inhibited cell cycle progression and DNA synthesis in primary human and established murine fibroblasts indicating that cyclin D2 can induce a non-proliferative state, possibly through sequestration of the CDK2 catalytic subunit (37). Moreover, E2F activity is tightly controlled by RB and deregulated expression or activity of most members of the E2F family has been detected in many human cancers (38). E2F5, similarly to E2F4, is necessary for pocket protein-mediated G1 arrest of cycling cells (39). Another gene of interest with an important role in cell cycle regulation found upregulated by C12 was CDC14B protein phosphatase. Cdc14B delays cell cycle progression from mitosis to S phase in an Skp2-dependent manner (40), while the Cdc14B-Cdh1-Plk axis inhibits Cdk1 and prevents entry into mitosis. The upregulation, therefore, of CCND2, E2F5 and CDC14B observed in our study could have played an important role in cell cycle response and survival to irradiation with C12 in A549 cells. Notably, overexpression of different members from both cyclin D and E2Fs family after irradiation with C12 has been detected before (11,22).

In conclusion, we aimed to identify genes whose expression was modified only by C12 and not by X-rays. In total, 49 mapped, network-eligible genes with known functions were characterized by gene ontology. Among others, two canonical pathways (aryl hydrocarbon receptor signalling and G1/S checkpoint machinery) were significant. The differential expression of key genes such as RARG, CCND1, E2F5 and CDC14B could be important in distinguishing the genetic signature and response of lung cancer cells to C12 therapy. Future studies are needed to examine these genes in detail and will provide insights into their role in lung cancer cells exposed to C12.

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