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RADIOSENSITIVITY OF HUMAN BRONCHIAL EPITHELIAL CELLS WITH GENETIC ALTERATIONS

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Purpose: To analyze the proliferation rates and the radiosensitivity of human bronchial epithelial cells (HBECs) with genetic alterations including human telomerase reverse transcriptase (hTERT), Cdk4 (cyclin dependent kinase 4), knockdown p53 and mutant K-ras overexpression.

Materials and Methods: HBECs obtained from human bronchus specimens were placed into short-term culture and were serially transfected with retroviral constructs containing Cdk4 and hTERT, resulting in continuously growing immortalized normal human bronchial epithelial cell lines. These cell lines were used to produce knock-down p53, mutant K-ras and knockdown p53 plus K-ras expressing cell lines; the proliferation rates and the radiosensitivity of these cell lines were evaluated. The control cells and the Cdk4, hTERT, knockdown p53 and K-ras expressing cells were irradiated (1 Gy-10 Gy) and the fraction survival was determined 1 week later.

Results: The proliferation rates of the cells expressing both knockdown p53 and K-ras were higher than those of the other cell lines and show a loss of contact inhibition in vitro. The HBECs infected with both knockdown p53 and mutant K-ras were more radioresistant compared to cells infected with only mutant K-ras or knockdown p53.

Conclusion: HBECs expressing knockdown p53 in addition to mutant K-ras have higher proliferation rates and additionally they lose the ability of growth arrest in response to DNA damage signals such as irradiation.

Key Words: Human Bronchial Epithelial Cells, Cdk4, Htert, P53, K-Ras, Irradiation.

GENETIK FARKLILIK GÖSTEREN İNSAN BRONŞ EPITEL HÜCRELERININ RADYOTERAPIYE HASSASIYETLERI

Amaç: Bu çalışmada, insan bronşiyal epitel hücrelerinin hTERT (insan telomeraz "reverse" transkriptazı), Cdk4 (siklin bağımlı kinaz 4), "knockdown" p53 ve mutant K-ras onkogeni ile enfekte edilmesiyle elde edilen hücre serilerinin hücre kültürü ortamında büyüme hızlarının ve radyoterapiye karşı hassasiyetlerinin karşılaştırılması amaçlanmıştır.

Metodlar: İnsan bronş dokusu örneklerinden elde edilen bronşiyal hücreler, öncelikle Cdk4 ve hTERT ile enfekte edilerek ölümsüzleştirildi. Daha sonra bu hücreler, "knockdown" p53, mutant K-ras ve "knockdown" p53 'le birlikte mutant K-ras eksprese eden hücre serilerinin elde edilmesinde kullanıldı.

Bulgular: Hem "knockdown" p53, hem de K-ras ekspresyonu gösteren hücrelerin büyüme hızlarının in vitro şartlarda diğer hücre serilerine oranla daha yüksek olduğu ve kontakt inhibisyon özelliklerini kaybettikleri saptandı. Kontrol hücreleri ile Cdk4, hTERT, "knockdown" p53 ve K-ras ile enfekte edilmiş olan hücrelere 1 Gy ile 10 Gy arasında farklı dozlarda radyoterapi uygulandı, 1 hafta sonra canlı kalan hücrelerin oranı belirlendi.

Sonuç: Bu çalışmanın sonuçları, hem"knockdown" p53 hem de mutant K-ras ile enfekte edilen bronşiyal epitel hücrelerinin, sadece K-ras veya "knockdown" p53 ile enfekte edilenlere oranla in vitro şartlarda yüksek proliferasyon hızına sahip olduklarını ve ayrıca DNA hasarına yol açan radyoterapiye cevap olarak büyüme duraklamasına girme özelliklerini kaybettiklerini ortaya koymaktadır.

Anahtar Kelimeler: İnsan Bronş Epitel Hücreleri, Cdk4, Htert, P53, K-Ras, Radyoterapi.

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Mutations in the p53 tumor suppressor gene and the K-ras oncogene have been frequently found in cancer patients, suggesting molecular explanations for tumor development and resistance to radiotherapy (1). The p53 tumor suppressor protein is a key mediator of an ATM-dependent DNA damage response cascade following cellular exposure to irradiation. The p53 gene is mutated or deleted in over 50% of all human cancers. In cancers without p53 mutations, p53 function can still be attenuated by cytoplasmic sequestration or degradation by viral oncoproteins such as HPV-E6 (2-4).

The Ras proteins are members of the small GTPase superfamily that regulate intracellular signaling by a cyclic process involving interconversion between active GTP-bound and inactive GDP-bound states, playing an essential role in controlling the activity of several crucial signaling pathways regulating normal cellular proliferation. It has been established that mutational activation of Ras genes can induce cancer in humans and Ras oncogenes are apparently involved in the early steps of carcinogenesis. K-Ras is an oncogene that has the ability to overcome cell-cell contact inhibition during proliferation and about 30% of human tumors contain an altered oncogenic K-Ras (5).

Cell-cycle events are controlled by cyclin-dependent kinases (CDKs), whose periodic activation is driven by cyclins. Tumorigenesis is the result of cell cycle disorganization, leading to uncontrolled cellular proliferation. Normally, these events are highly conserved due to the existence of conservatory mechanisms and molecules such as cell cycle genes and their products: cyclins, cyclin dependent kinases (Cdks), Cdk inhibitors (CKI) and extracellular factors such as growth factors (6). Cyclin-dependent kinase 4 (Cdk4) plays a crucial role in the regulation of the progression through the G1 phase and also has an impact on G1/S transition (6-8). Additionally, in epithelial systems, the p16/RB pathway may function as a stress senescence-signaling pathway independent of telomere shortening. Overexpressing cyclin-dependent kinase 4 (Cdk4) in human epidermal keratinocytes and human mammary epithelial cells prevents the p16(INK4a)-associated premature growth arrest due to telomere-independent stress such as inadequate culture conditions (8).

The telomere hypothesis postulates that progressive shortening of the ends of chromosomes occurs in the absence of telomerase, and telomerase is considered the mitotic clock that regulates the onset of replicative senescence in normal somatic cells. hTERT is the key determinant of the enzymatic activity and its transcriptional control is a major contributor to the regulation of telomerase activity in many types of tumors. Telomerase is shown to have a correlation with cell cycle progression, which is controlled by the regulation of cyclins, cyclin-dependent kinases (cdks) and cyclin-dependent kinase inhibitors (8-9).

In this study, our aim was to analyze the proliferation rates and radiosensitivity of human bronchial epithelial cells (HBECs) with genetic alterations including human telomerase reverse transcriptase (hTERT), Cdk4 (cyclin-dependent kinase 4), knockdown p53 and mutant K-ras overexpression.

MATERIALS AND METHODS

Primary Culture of Human Bronchial Epithelial Cells: The bronchial tissue from a 36-year-old lung transplant patient was transported to the cell culture laboratory in sterile 1xPBS on ice. The tissue was washed with sterile 1xPBS, cut into small pieces and incubated with 4 ml of dispase (Sigma) solution at 4oC overnight. The next day, the tube was mixed by vortex, and the dispase solution was aspirated and transferred to another tube. The rest of the tissues were digested with 1 ml of trypsin (Sigma) for 30 min at 37oC in a water bath. Following the addition of 100 µl of trypsin inhibitor, the supernatant was transferred to the dispase tube and spun (2000g) for 5 min. The supernatant including the cells were plated on collagen-coated (1%) plates in KSFM (Gibco) under low O2 conditions at 370C. Each week the cell lines were collected, counted and replated. The population doubling (PD) was calculated each week by the following formula: log (number of cells collected/number of cells initially plated)/log 2.

Retroviral Vector Construction and Infection: We established immortal human bronchial epithelial cells by introducing mouse cyclin-dependent kinase 4 (Cdk4) and human telomerase reverse transcriptase (hTERT) into normal bronchial epithelial cells. Primary HBECs (PD:3) were infected with medium containing viral supernatant produced from pSRαMSU expressing the Cdk4 vector in the presence of 4 µg/ml Polybrene (Sigma) for 10 to 12 hours. Cells were allowed to recover for 72 hours, followed by 30 µg/ml gentamycin selection (Calbiochem) for 10 days. Bronchial epithelial cells (PD:10) were then infected with a second retroviral construct, pBABE expressing hTERT in the presence of 4 µg/ml Polybrene (Sigma), followed by selection with 250 ng/ml puromycin (Sigma) for 3 to 4 days, initiated 72 hours later.

Using this immortalized cell line we produced knockdown p53 and mutant K-ras expressing bronchial epithelial cells. pSRZ-p53 vector was used to obtain p53 knockdown cells, whereas pSUPER.retro (pSR) was used to produce stable and specific knockdown of oncogenic K-ras. The vectors were obtained from University of Texas Southwestern and they were used with 4 µl/ml Polybrene (Sigma) for the infection of HBECs expressing Cdk4 and hTERT.

Determination of telomerase activity: TRAP assay was used to show telomerase activity as described by Kim et al. (10); 1x105 cells were collected and lysed in 40 µl NP-40 lysis buffer (10 mM Tris-HCl pH 8.0, 1.0 mM MgCl2, 1 mM EDTA, 1% NP-40, 0.25 mM sodium deoxycholate, 10% glycerol, 150 mM NaCl, 5 mM β-mercaptoethanol, 0.1 mM AEBSF) for 30 min on ice, and 1 µl of total cellular lysate (2500 cells) was used for each reaction. Samples were analyzed with TRAP-eze kit reagents and were exposed to 30 minutes of telomerase extension at 30ºC. Telomerase was inactivated at 94°C for 90 seconds and the extension products were amplified by 28 PCR cycles (94°C for 30 sec, 52°C for 30 sec, 72°C for 45 sec) in the presence of a Cy5-labeled TS primer (5'-Cy5-AATCCGTCGAGCAGAGTT) (Integrated DNA Technologies, Inc.). A549 lung cancer cells were used as a positive control, whereas lysis buffer was used as a negative control for each run. PCR samples (20 µl) were run on a 10% non-denaturing acrylamide gel in 0.5 x TBE at 250 V for 2.5 hrs. Gels were scanned using a STORM 860 PhosphorImager scanner system (Molecular Dynamics).

Exposure to Irradiation: The control group of HBECs and the Cdk4, hTERT, knockdown p53 and mutant K-ras expressing HBECs were plated 100x103 cell/plate. Following their attachment (24-48 hrs), fresh media were given and they were irradiated with 1-10 Gy at a dose rate of 0.63 Gy/min (Theratron 780-C, Philips Medizin Systeme) at room temperature (200C). The plates were incubated at 370C for 5 days without changing the media and the living cells were counted by Beckmann Coulter at the end of this period.

RESULTS

The proliferation rate and the final density of the control HBECS and the immortalized cells with four different genetic alterations were evaluated: Cdk4+hTERT, Cdk4+hTERT+p53 knockdown, Cdk4+hTERT+mutant K-Ras and Cdk4+hTERT+p53 knockdown+mutant K-Ras. Knockdown p53 expressing, mutant K-Ras expressing and p53RNAi+mutant K-Ras expressing cells had higher growth rate compared to immortal and primary HBECs. The prolife-

Figure 1. Determination of telomerase activity by TRAP: Lane 1.hTERT (+) HBEC, Lane 2.Cdk4 (+) HBEC, Lane 3. hTERT and Cdk4 (+) HBEC, Lane 4. Control (HBEC), Lane 5. Negative control (Lysis buffer), Lane 6. Positive control (A549 lung cancer cell line).

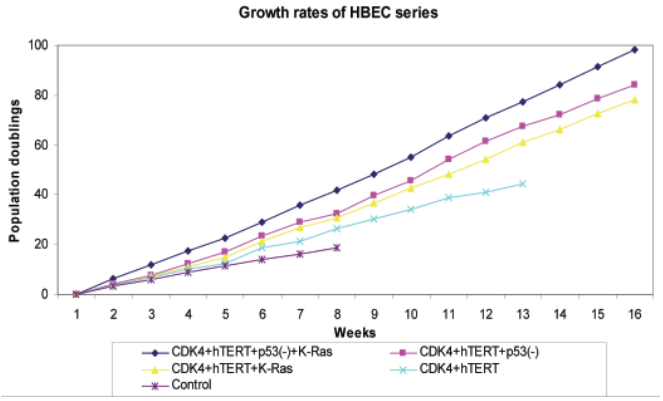

Figure 2. Growth rates of HBEC series with different genetic alterations.

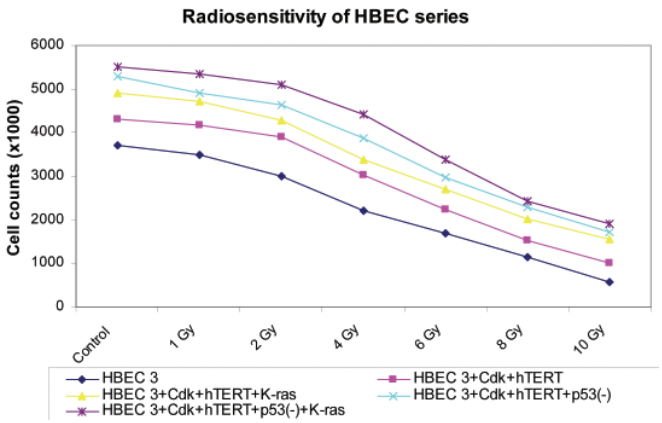

Figure 4. Radiosensitivity of HBEC cells with different genetic alterations.

ration rate of the cells expressing both knockdown p53 and mutant K-Ras were significantly higher than the other cell types at each split. The growth rates of the HBEC series were compared by the population doublings (PD) as shown in Fig. 2. The cells expressing Cdk4 and hTERT bypassed growth arrest and telomere dependent senescence, immortalized and continuously replicated, exceeded 45-50 PD, whereas the life span of primary HBECs stopped at around 14-18 PD.

In our study, we observed some morphologic changes after these genetic alterations. Cdk4 and hTERT expressing cells also showed a loss of collagen-coated surface dependence in addition to low O2 incubators, which are essential for primary bronchial cells. Additionally, both mutant K-Ras and p53 knockdown cells lost contact inhibition partially as well as the mutant K-Ras+p53 knockdown expressing cells. The morphology of the control HBECs and the cells with expressing hTERT+Cdk4+mutant K-Ras+knockdown p53 is given in Fig. 3A and 3B.

The radiosensitivity of the HBEC series with different genetic alterations is given in Fig.4. Our results suggest that primary HBECs and HBECs immortalized by overexpression of Cdk4 + hTERT are the most radiosensitive cell lines. HBECs infected with knockdown p53+ mutant K-ras were found to be most radioresistant cell line. The combination of knockdown p53 and mutant K-ras makes the cells more radioresistant than the cells infected with only mutant K-ras or knockdown p53.

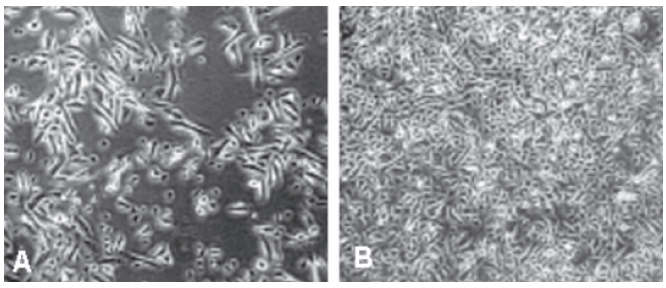

Figure 3. Primary bronchial epithelial cells (A), Cdk4 + hTERT + 'knockdown' p53+K-Ras expressing bronchial epithelial cells (B).

DISCUSSION

Radiotherapy alone or in combination with chemotherapy can be curative for a number of tumor sites. Typical radiocurative regimens require a series of individual fractions of 1.8-2 Gy over 6 to 8 weeks (i.e. total doses in the order of 60-80 Gy) in which fractionation maximizes the cell killing in tumor cells. The final level of cell killing during radiotherapy is due to multiple factors, which can be summarized as the five R's of radiotherapy: intrinsic radiosensitivity, redistribution of cells within the cell cycle, the repair capacity of both normal and tumor cells, reoxygenation and repopulation of the cells during radiotherapy. The epithelial human tumors treated with radiotherapy are reported to undergo radiation-induced terminal growth (tumor cell senescence) or mitotic catastrophe, whereas isogenic cells that lack p53 function are relatively radioresistant due to inhibition of p53-dependent apoptosis (11,12).

A large number of genes involved in controlling the malignant transformation of human bronchial epithelial cells (HBECs), as a result of either qualitative or quantitative alterations in expression, have been identified in the last ten years. The ability of mutated active K-Ras to modulate the activity of signaling pathways, following exposure to ionizing radiation, is still unknown (13). p53 readily facilitates radiation induced apoptosis in radiosensitive cells such as lymphocytes, thymocytes, and germ cells. Additionally, in fibroblasts and epithelial cells, p53 acts to induce cell cycle arrest in G1 and G2 phases of the cell cycle to prevent cells from proliferating under conditions of DNA damage and repair. It is reported that a loss of p53 function due to mutation or degradation causes alterations in G1 and G2 cell cycle check point control, cell death, DNA repair and genetic stability. The results of these alterations can cause generation of radioresistant mutant tumor cells and decreased chemosensitivity in vitro (12).

In the present study, we reproducibly immortalized HBECs from routine cultures of bronchial specimens using Cdk4 and hTERT expression vectors without introducing viral oncoproteins. Ectopic Cdk4 was introduced to eliminate the stress response of the bronchial epithelial cells to the in vitro

culture conditions, followed by the introduction of hTERT to prevent further telomere shortening (14,15). The combination of knockdown p53 and mutant K-ras caused partial loss of contact inhibition and made the cells more radioresistant than the cells infected with only mutant K-ras or knockdown p53. HBECs lost the dependence of collagen-coated plates and low O2 cell culture conditions after they began to express Cdk4+hTERT but they were still radiosensitive compared to knockdown p53 and mutant K-ras expressing cells.

Although we report here that HBECs with four genetic alterations, including hTERT and Cdk4 overexpression, inactivation of p53 and mutant K-ras, lose the ability of contact inhibition, Ramirez et al. (16) showed that using these four genetic alterations is not sufficient for HBECs to transform to cancer. Additional genetic alterations are required for malignant transformation of human bronchial epithelial cells.

In summary, HBEC lines with defined genetic changes are a valuable new tool for studying the molecular pathogenesis of lung cancer in addition to the differentiation and radiosensitivity of bronchial epithelial cells.

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REFERENCES

1. Huber RM, Stratakis DF. Molecular oncology—perspectives in lung cancer. Lung Cancer 2004; 45: 209-213.
2. Finlay CA, Hinds PW, Levine AJ. The p53 protooncogene can act as a suppressor of transformation. Cell 1989; 57: 1083-1093.
3. Hainaut P, Hernandez T, Robinson A, Rodriguez-Tome P, Flores T, Hollstein M, Harris CC, Montesano R. IARC database of p53 gene mutations in human tumors and cell lines: Updated compilation, revised formats and new visualisation tools. Nucleic Acids Res 1998; 26: 205-213.
4. Sherr CJ, Weber JD. The ARF/p53 pathway. Curr Opin Genet Dev 2000; 10: 94-99.
5. Colombo S, Peri F, Tisi R, Nicotra F, Martegani E. Design and characterization of a new class of inhibitors of ras activation. Ann N Y Acad Sci 2004; 1030: 52-61.
6. Golias CH, Charalabopoulos A, Charalabopoulos K. Cell proliferation and cell cycle control: a mini review. Int J Clin Pract. 2004; 58: 1134-1141.
7. Lange S, Viergutz T, Simko M. Modifications in cell cycle kinetics and in expression of G1 phase-regulating proteins in human amniotic cells after exposure to electromagnetic fields and ionizing radiation. Cell Prolif. 2004; 37: 337-349.
8. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. Nature 1990; 345: 458-460.
9. Harley CB, Vaziri H, Counter CM, Allsopp RC. The telomere hypothesis of cellular aging. Exp Gerontol. 1992; 27: 375-382.
10. Kim NW, Piatyszek MA, Prowse KR, et al. Specific association of human telomerase activity with immortal cells and cancer. Science 1994; 266: 2011-2015.
11. Andrew R. Cuddihy, Robert G. Bristow. The p53 protein family and radiation sensitivity: Yes or no? Cancer and Metastasis Reviews 2004; 23: 237-257.
12. Bristow RG, Benchimol S, Hill RP. The p53 gene as a modifier of intrinsic radiosensitivity: Implications for radiotherapy. Radiother Oncol 1996; 40: 197-223.
13. DeMuth JP, Jackson CM, Weaver DA, Crawford EL, Durzinsky DS, Durham SJ, Zaher A, Phillips ER, Khuder SA, Willey JC. The gene expression index c-myc x E2F-1/p21 is highly predictive of malignant phenotype in human bronchial epithelial cells. Am J Respir Cell Mol Biol. 1998; 19: 18-24.
14. Ramirez RD, Morales CP, Herbert BS, Rohde JM, Passons C, Shay JW, Wright WE. Putative telomere-independent mechanisms of replicative aging reflect inadequate growth conditions. Genes Dev 2001; 15: 398-403.
15. Ramirez RD, Herbert BS, Vaughan MB, Zou Y, Gandia K, Morales CP, Wright WE, Shay JW. Bypass of telomere-dependent replicative senescence (M1) upon overexpression of Cdk4 in normal human epithelial cells. Oncogene 2003; 22: 433-444.
16. Ramirez RD, Sheridan S, Girard L, Sato M, Kim Y, Pollack J, Peyton M, Zou Y, Kurie JM, Dimaio JM, Milchgrub S, Smith AL, Souza RF, Gilbey L, Zhang X, Gandia K, Vaughan MB, Wright WE, Gazdar AF, Shay JW, Minna JD. Immortalization of human bronchial epithelial cells in the absence of viral oncoproteins. Cancer Res. 2004; 64: 9027-9034.