Austin Journal of Cancer and Clinical Research

Open Access

Austin Publishing Group

Research Article

Strong Down-Regulation of Tumor Suppressor Genes *RB1* and *CTDSPL* is Associated with Aberrant Expression of Cell Cycle Regulation Genes in Non-Small Cell Lung Cancer

Senchenko VN[1]*, Dmitriev AA[1], Krasnov GS[1], Kudryavtseva AV[1], Dardyk MV[2], Beniaminov AD[1] and Kondratieva TT[2]

[1]Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Russia

[2]N.N. Blokhin Russian Cancer Research Center, Russian Academy of Medical Sciences, Russia

***Corresponding author:** Senchenko VN, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Vavilova 32, 119991 Moscow, Russia

Received: June 01, 2015; **Accepted:** August 05, 2015; **Published:** August 08, 2015

Abstract

The search of specific molecular markers for differential diagnostics and new targets for combined targeted therapy or gene therapy is one of the key goals of molecular oncology. The first step in this direction is a comparative expression analysis of genes involved in a complex network of interactions of cell cycle control molecules in tumors. In the present study, advanced quantitative expression analysis of 84 genes (Human Cell Cycle Regulation Panel, Roche) revealed dysfunctions of $p16^{INK4A}$–Cdk/cyclin D1–Rb and p53/$p21^{Waf1}$ pathways associated with strong down-regulation of two tumor-suppressor genes, *RB1* and *CTDSPL*, in non-small cell lung cancer (NSCLC). Rb protein, a key regulator of cell cycle, can be activated by small CTD-serine phosphatase CTDSPL/RBSP3. The analysis revealed that over-expression of many genes from the panel was stronger in lung adenocarcinoma (ADC) than squamous cell carcinoma (SCC) and more pronounced in metastatic tumors. A number of genes showed expression alterations which were specific to NSCLC histological type or metastases presence. These genes could be potential NSCLC biomarkers. Twenty five genes (survivin, cyclins, Ser/Thr-protein kinases, transcription factors, phosphatases, *etc.*) with the strongest expression gains (up to 100-fold) could be potential targets for future biotherapy approach of NSCLC (both ADC and SCC). Our data concerning incremental deregulation of *RB1, CTDSPL* and other cell cycle control genes are in flow with the continuum model for tumor suppression.

Keywords: Non-small cell lung cancer; Gene expression; Tumor suppressor gene; Cell cycle control gene; Biomarker; Target for biotherapy

Introduction

Multidisciplinary approach for a non-small cell lung cancer (NSCLC) diagnostics allows to choose the most adequate treatment strategy [1]. Particular relevance acquired differential diagnosis of lung adenocarcinoma (ADC) and squamous cell carcinoma (SCC) that requires specific markers for each histological type [2]. In case of ADC, targeted therapy has a great success and is one of the perspective treatment directions [3]. However, targeted therapy, aimed at individual targets, is often accompanied by the rapid development of drug resistance [3]. A new generation of anti-cancer drugs for combined targeted therapy and gene therapy involves the search for not only individual, but a number of targets at once for simultaneous blocking or restoring of their activity. In this regard, a concurrent expression analysis of many genes in a complex network of interactions of cell cycle control elements is currently relevant.

Deregulation of cell cycle control is recognized as the hallmark of carcinogenesis, including NSCLC. Numerous works were focused on aberrations of gene expression caused by genetic and epigenetic mechanisms for one or more of the key elements of cell cycle regulation in primary lung tumors [4,5]. However, only a few studies compared multiple expression markers among cell control elements and their value for a clinical use simultaneously.

The Rb protein encoded by *RB1* gene (retinoblastoma 1, *RB*, *pRb*; 13q14.2) is a key component of the $p16^{ink4A}$–Cdk/cyclin–Rb pathway responsible for cell division arrest at the G_1/S checkpoint [6]. The lack of Rb activity correlated with lower survival of patients with various types of lung cancer [7,8]. The *CTDSPL* gene (also known as *RBSP3*; 3p21.3) belongs to the gene family of small CTD (carboxy-terminal domain) serine phosphatases that preferentially catalyzes serine-5 dephosphorylation in the consensus repeat sequence of the RNA polymerase II (Pol II) largest subunit and other proteins. In case of Pol II, this modification leads to the negative regulation of transcriptional activity [9-11]. Another function of CTDSPL is believed to be an activation of the phosphorylated precursor of Rb by dephosphorylation of serine 807/811 [11].

In the present study, advanced quantitative expression analysis revealed strong down-regulation of two tumor suppressor genes (TSGs), *RB1* and *CTDSPL*, associated with aberrant expression of 84 genes (Human Cell Cycle Regulation Panel, Roche) from $p16^{INK4A}$–Cdk/cyclin D1–Rb and p53/$p21^{Waf1}$ pathways in non-small cell lung

Citation: Senchenko VN, Dmitriev AA, Krasnov GS, Kudryavtseva AV, Dardyk MV, Beniaminov AD, et al. Strong Down-Regulation of Tumor Suppressor Genes *RB1* and *CTDSPL* is Associated with Aberrant Expression of Cell Cycle Regulation Genes in Non-Small Cell Lung Cancer. Austin J Cancer Clin Res 2015; 2(7): 1062.

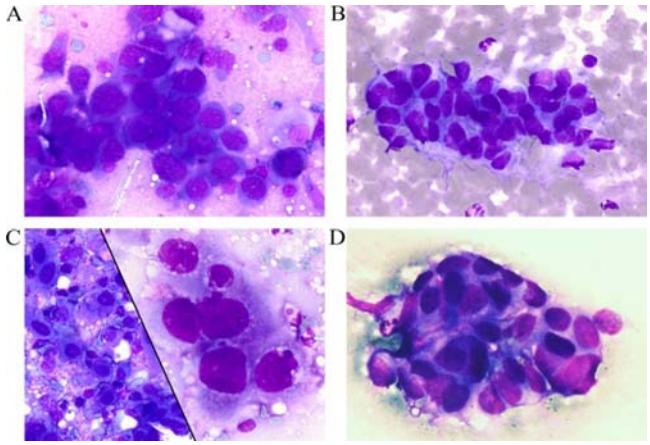

Figure 1: Cytological investigation of lung cancer bronchoscopy material (Leishman stain, azur-eosin). **A.** Moderately differentiated adenocarcinoma cells with intracellular mucus (x1000). **B.** Cells of well differentiated adenocarcinoma. Irregular nuclear shape and mitosis are determined (x600). **C**. Squamous cell carcinoma. Moderately differentiated cancer cells with abundant cytoplasm and keratinization features around the nuclei (two fragments are represented: left – x400, right – x1000). **D**. Keratinizing squamous cell carcinoma. Groups of poorly differentiated cancer cells with the high nuclear-cytoplasmic ratio are observed (x1000).

cancer (ADC and SCC). Obtained data allowed to suggest novel potential differential diagnostics markers and targets for future biotherapy approach of NSCLC.

Materials and Methods

Tissue samples

Primary NSCLC (ADC and SCC) and the adjacent morphologically normal tissues were obtained from patients after surgical resection of tumors prior radiation or chemotherapy and characterized according to the International TNM Classification system [12] at the N.N. Blokhin Russian Cancer Research Center (Moscow, Russia). The clinical diagnosis was confirmed by pathomorphological examination at the Department of Tumor Pathologic Anatomy, Research Institute for Clinical Oncology (Moscow, Russia). Approval of the use of all human tissues was obtained from the Ethics Committee of N.N. Blokhin Russian Cancer Research Center. The patients gave written informed consent that is available upon request. The study was done in accordance with the principles outlined in the Declaration of Helsinki.

RNA Isolation and Reverse transcription

RNA isolation was performed using the RNeasy Mini Kit (Qiagen, Netherlands) according to the manufacturer's protocol. The quantity and quality of the RNA was determined using NanoDrop ND-1000 (NanoDrop Technologies Inc., USA). The reverse transcription reaction was carried out using M-MuLV Reverse Transcriptase (Fermentas, Lithuania) according to the manufacturer's instructions.

Quantitative PCR

The preliminary expression analysis of two TSGs, *CTDSPL* and *RB1*, was done in 14 ADC and 16 SCC samples. QPCR was performed with commercial primers and TaqMan probes using a 7500 Real-Time PCR System (Applied Biosystems, USA). Then simultaneous expression profiling of 84 genes involved in a cell cycle regulation was performed in ADC and SCC samples with the use of a LightCycler 480 PCR System (Roche, Switzerland) and "Human Cell Cycle Regulation Panel" (Roche) according to the manufacturer's protocol. These quantitative data were analyzed using three reference genes *GAPDH*, *HPRT1* and *YWHAZ* and the relative quantification or ΔΔCt-method. All calculations were performed using our program ATG ("Analysis of Transcription of Genes") [13,14]. At least 2-fold mRNA level changes were considered as significant because of mRNA level variability of the reference genes.

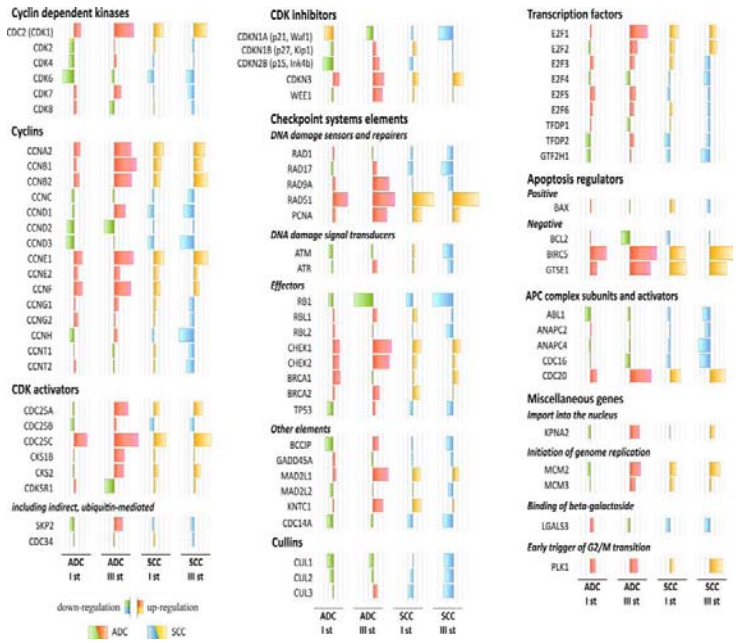

Figure 2: Expression profiles of 84 genes involved in a cell cycle. Four NSCLC samples were analyzed (from left to right): ADC stage I ($T_1N_0M_0$) and stage III ($T_2N_3M_0$); SCC stage I ($T_2N_0M_0$) and stage III ($T_2N_2M_0$). Red and yellow – up-regulation, green and blue – down-regulation.

Table 1: The most considerable relative mRNA level changes (increase and decrease, n-fold) of the cell cycle regulation genes in ADC and SCC.

Gene symbol	Expression ratio, n-fold			Gene name
	ADC III/I	SCC III/I	ADC/ SCC	
Negative apoptosis regulators				
GTSE1	8 ↑			G2 and S phase-expressed protein 1 (B99 homolog)
BIRC5	5 ↑	3 ↑		Baculoviral IAP repeat-containing protein 5 (Apoptosis inhibitor survivin)
Cyclins				
CCNA2	5 ↑			Cyclin-A2 (Cyclin-A)
CCNB1	18 ↑			G2/mitotic-specific cyclin-B1
CCNB2	6 ↑	3 ↑		G2/mitotic-specific cyclin-B2
CCND1	7 ↑		9 ↑	G1/S-specific cyclin-D1 (PRAD1 oncogene)
CCND2			3 ↓	G1/S-specific cyclin-D2
CCNE1	5 ↑			G1/S-specific cyclin-E1
CCNF	3 ↑			G2/mitotic-specific cyclin-F
CCNT1		3 ↓		Cyclin-T1 (CycT1)
CCNT2		4 ↓		Cyclin-T2 (CycT2)
Cyclin-dependent kinases (CDK)				
CDC2	7 ↑			Cell division control protein 2 homolog (CDK1)
CDK7			3 ↑	Cell division protein kinase 7
APC complex subunits and activators				
ANAPC4		4 ↓		Anaphase-promoting complex subunit 4 (APC4)
CDC20	9 ↑			Cell division cycle protein 20 homolog (p55CDC)
CDK activators				
CDC25A	9 ↑			M-phase inducer phosphatase 1
CDC25C	5 ↑			M-phase inducer phosphatase 3
CDK5R1	5 ↓			Cyclin-dependent kinase 5 activator 1 precursor (CDK5 activator 1)
CDK inhibitors				
CDKN1A		6 ↓		Cyclin-dependent kinase inhibitor 1 (p21)
Checkpoint system elements				
ATM		6 ↓		Serine-protein kinase ATM
CHEK1	6 ↑			Serine/threonine-protein kinase Chk1
PCNA	5 ↑			Proliferating cell nuclear antigen (PCNA)
RAD17			4 ↑	Cell cycle checkpoint protein RAD17 (hRad17)
RAD51	3 ↑			DNA repair protein RAD51 homolog 1 (hRAD51)
MAD2L1	6 ↑			Mitotic spindle assembly checkpoint protein MAD2A (MAD2-like 1)
Transcription factors				
E2F1	11 ↑			Transcription factor E2F1 (E2F-1)
E2F2	4 ↑			Transcription factor E2F2 (E2F-2)
E2F3		3 ↓		Transcription factor E2F3 (E2F-3)
E2F5			3 ↑	Transcription factor E2F5 (E2F-5)
Miscellaneous genes				
MCM2	7 ↑			DNA replication licensing factor MCM2 (Minichromosome maintenance protein 2 homolog)
PLK1		4 ↑		Serine/threonine-protein kinase PLK1
CUL3			4 ↑	Cullin-3 (CUL-3)

Note: Here genes with 3-fold (and more) mRNA level changes are represented (ADC and SCC stage III *vs.* stage I; ADC *vs.* SCC); ↑ – increase; ↓ – decrease.

Results and Discussion

In the present study we compared expression profiles of two tumor suppressor genes, *RB1* and *CTDSPL,* in the same primary lung tumors (NSCLC: ADC and SCC) to find mutually interdependent changes and their association with tumor characteristics. At the beginning, we revealed noticeable mRNA level decrease of both *RB1* and *CTDSPL* in the majority of NSCLC samples (data not shown). The down-regulation was more pronounced in tumors with metastasis to the regional lymph nodes compared to metastasis-free tumors. We chose four samples for further comparison: moderately differentiated ($T_1N_0M_0$) and well differentiated ($T_2N_3M_0$) ADC (Figure 1A and 1B respectively); moderately differentiated ($T_2N_0M_0$) and poorly differentiated ($T_2N_2M_0$) SCC (Figure 1C and 1D respectively). These two pairs of ADC and SCC samples were characterized by the strongest decrease of the mRNA level in samples with metastases compared to those without metastasis: for *CTDSPL* – 3.4-fold *vs.* 2.1 (ADC) and 94-fold *vs.* 7.5 (SCC); for *RB1* – 16-fold *vs.* 2.1 (ADC) and 19-fold *vs.* 2.7 (SCC). Then expression profiling was performed to reveal the association between the down-regulation of *RB1* and *CTDSPL* and aberrations of 84 genes encoding cell cycle control elements of two basic signaling pathways, Rb (Rb, cyclin D1, $p16^{INK4A}$) and p53/$p21^{Waf1}$, in the same pairs of ADC and SCC samples. In general, detected expression profiles abnormalities of examined cell cycle regulation genes (Figure 2) were reflected in different degrees of

cell differentiation and severity of the cell nuclei atypia (Figure 1) in two major histological types of NSCLC.

The comparison of expression data revealed the following important features (Figure 2**,** Table 1):

1. Over-expression of many genes from the panel was stronger in lung ADC than SCC and more pronounced in metastatic tumors.

2. For 19 genes we showed 3-18-fold up-regulation in the metastatic ADC compared to metastasis-free. Six genes were down-regulated in the metastatic SCC compared to non-metastatic one. The expression level was 3-6-fold higher in both metastatic ADC and SCC compared to non-metastatic for only two genes – survivin (*BIRC5*) and cyclin – B2 (*CCNB2*). (Potential markers of metastatic activity of ADC and/or SCC).

3. Six genes – *CCND1* and *CCND2* (cyclins D1 and D2), *CDK7* (cyclin-dependent kinase), *RAD17* (element checkpoints), *E2F5* (transcriptional factor) and *CUL3* (cullin) showed alterations in expression specific to NSCLC subtype. The *CCND2* mRNA level was 3-fold higher in SCC compared to ADC. The expression levels of other genes were in a 3-9 times higher in ADC compared to SCC. (Potential markers of discrimination between ADC and SCC).

4. The highest mRNA level was observed for the following genes: *CDC2* (cyclin-dependent kinase, CDK), *CCNA2* and *CCNE1* (cyclins A2 and E1), *CDC20* (activator anaphase-promoting complex, APC) and *CDC25C* (activators CDK), *RAD51* and *MAD2L1* (elements mechanism checkpoints), *BIRC5* and *GTSE1* (inhibitor of apoptosis), *PLK1* (APC activator) in all samples regardless NSCLC subtype and the presence of metastases. (Potential targets for combined therapy of NSCLC – ADC and SCC).

The next efforts in this research direction will help to develop a new optimal gene panel for searching of general and specific targets for the treatment of NSCLC subtypes. These problems are particularly acute in connection with the relevance of accurate ADC and SCC detection because erroneous diagnosis and choice of therapy can cost a patient's life [15,16].

The expression analysis of the genes participating in a cell cycle progression revealed evident dysfunctions of Rb/E2F and other pathway checkpoints in NSCLC while the essential impact comes from the *RB1* and *CTDSPL* silencing. The summary of currently available data includes: a) an inhibition of *RB1* gene expression at the transcriptional level and, probably, via microRNA interference takes place in different cancers [17]; b) the dramatic increase of expression of cyclins E and others cyclins, also known to be up-regulated in various types of cancer [18] can accelerate Rb phosphorylation by Cyclin-CDK complexes; c) p21, an inhibitor of these complexes, may be under-expressed [19]; d) transfection of tumor cells with *CTDSPL* can lead to the content decrease of inactive phosphorylated form of Rb [11]; and e) *CTDSPL* down-regulation can decline miR-26a-mediated suppression of CDK6, cyclins D2, E1, E2 expression [20].

Pronounced character of expression abnormalities of two tumor suppressors – CTDSPL and its target Rb, along with various cell cycle control genes in NSCLC supports the new continuum model of tumor suppression [21], in which authors integrated "two-hit" model of carcinogenesis with a hypothesis of subtle dosage effects of tumor suppressors. It underlines the importance of TSG expression attenuation. Our results propose that a decline of tumor suppression is resulted from a gradual falling of TSG expression even without dramatic loss of alleles: dozens of slight aberrations in various genes may lead to significant tumor promotion effect.

Conclusion

We showed expression deregulation of the majority of cell cycle regulation genes involved in associated with down-regulation of two tumor suppressor genes *RB1* and *CTDSPL*. Six genes (*CCND1*, *CCND2*, *CDK7*, *RAD17*, *E2F5*, and *CUL3*) showed subtype-specific alterations of their expression and were suggested potential markers of NSCSL subtype – ADC or SCC. Two genes *BIRC5* and *CCNB2* revealed metastasis-specific expression up-regulation and can be speculated as markers of NSCLC aggressiveness. Many genes (survivin, cyclins, Ser/Thr-protein kinases, etc.) with expression gains (up to 100-fold) could be potential targets for future biotherapy approach of NSCLC – especially four genes (*BIRC5*, *CCNE1*, *CDC25C*, and *RAD51*) that had high expression level in all studied tumors independently of particular NSCLC subtype, and presence of metastases.

References

1. Travis WD, Brambilla E, Noguchi M, Nicholson AG, Geisinger KR, Yatabe Y, et al. International association for the study of lung cancer/american thoracic society/european respiratory society international multidisciplinary classification of lung adenocarcinoma. J Thorac Oncol. 2011; 6: 244-285.

2. Spiro SG, Tanner NT, Silvestri GA, Janes SM, Lim E, Vansteenkiste JF, et al. Lung cancer: progress in diagnosis, staging and therapy. Respirology. 2010; 15: 44-50.

3. Gower A, Wang Y, Giaccone G. Oncogenic drivers, targeted therapies, and acquired resistance in non-small-cell lung cancer. J Mol Med (Berl). 2014; 92: 697-707.

4. Vincenzi B, Schiavon G, Silletta M, Santini D, Perrone G, Di Marino M, et al. Cell cycle alterations and lung cancer. Histol Histopathol. 2006; 21: 423-435.

5. Yokota J, Kohno T. Molecular footprints of human lung cancer progression. Cancer Sci. 2004; 95: 197-204.

6. Weinberg RA. The retinoblastoma protein and cell cycle control. Cell. 1995; 81: 323-330.

7. Osada H, Takahashi T. Genetic alterations of multiple tumor suppressors and oncogenes in the carcinogenesis and progression of lung cancer. Oncogene. 2002; 21: 7421-7434.

8. Husgafvel-Pursiainen K, Boffetta P, Kannio A, Nyberg F, Pershagen G, Mukeria A, et al. p53 mutations and exposure to environmental tobacco smoke in a multicenter study on lung cancer. Cancer Res. 2000; 60: 2906-2911.

9. Yeo M, Lee SK, Lee B, Ruiz EC, Pfaff SL, Gill GN, et al. Small CTD phosphatases function in silencing neuronal gene expression. Science. 2005; 307: 596-600.

10. Yeo M, Lin PS, Dahmus ME, Gill GN. A novel RNA polymerase II C-terminal domain phosphatase that preferentially dephosphorylates serine 5. J Biol Chem. 2003; 278: 26078-26085.

11. Kashuba VI, Li J, Wang F, Senchenko VN, Protopopov A, Malyukova A, et al. RBSP3 (HYA22) is a tumor suppressor gene implicated in major epithelial malignancies. Proc Natl Acad Sci U S A. 2004; 101: 4906-4911.

12. Travis WD, Brambilla E, Muller-Hermelink HK, Harris CC. World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Lung, Pleura, Thymus and Heart. Lyon: IARC Press. 2004.

13. Krasnov GS, Oparina NIu, Dmitriev AA, Kudriavtsev AV, Anedchenko EA, Kondrat'eva TT, et al. [Novel reference gene RPN1 for normalization of

quantitative data in lung and kidney cancer]. Mol Biol (Mosk). 2011; 45: 238-248.

14. Senchenko VN, Krasnov GS, Dmitriev AA, Kudryavtseva AV, Anedchenko EA, Braga EA, et al. Differential expression of CHL1 gene during development of major human cancers. PLoS One. 2011; 6: e15612.

15. Russell PA, Wainer Z, Wright GM, Daniels M, Conron M, Williams RA. Does lung adenocarcinoma subtype predict patient survival?: A clinicopathologic study based on the new International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society international multidisciplinary lung adenocarcinoma classification. J Thorac Oncol. 2011; 6: 1496-1504.

16. Fischer AH, Cibas ES, Howell LP, Kurian EM, Laucirica R, Moriarty AT, et al. Role of cytology in the management of non-small-cell lung cancer. J Clin Oncol. 2011; 29: 3331-3332.

17. Di Fiore R, D'Anneo A, Tesoriere G, Vento R. RB1 in cancer: different mechanisms of RB1 inactivation and alterations of pRb pathway in tumorigenesis. J Cell Physiol. 2013; 228: 1676-1687.

18. Akli S, Keyomarsi K. Cyclin E and its low molecular weight forms in human cancer and as targets for cancer therapy. Cancer Biol Ther. 2003; 2: S38-47.

19. Komiya T, Hosono Y, Hirashima T, Masuda N, Yasumitsu T, Nakagawa K, et al. p21 expression as a predictor for favorable prognosis in squamous cell carcinoma of the lung. Clin Cancer Res. 1997; 3: 1831-1835.

20. Zhu Y, Lu Y, Zhang Q, Liu JJ, Li TJ, Yang JR, et al. MicroRNA-26a/b and their host genes cooperate to inhibit the G1/S transition by activating the pRb protein. Nucleic Acids Res. 2012; 40: 4615-4625.

21. Berger AH, Knudson AG, Pandolfi PP. A continuum model for tumour suppression. Nature. 2011; 476: 163-169.

Austin J Cancer Clin Res - Volume 2 Issue 7 - 2015
ISSN : 2381-909X | www.austinpublishinggroup.com

Citation: Senchenko VN, Dmitriev AA, Krasnov GS, Kudryavtseva AV, Dardyk MV, Beniaminov AD, et al. Strong Down-Regulation of Tumor Suppressor Genes *RB1* and *CTDSPL* is Associated with Aberrant Expression of Cell Cycle Regulation Genes in Non-Small Cell Lung Cancer. Austin J Cancer Clin Res 2015; 2(7): 1062.