

Clinical significance of insulin receptor substrate-1 down-regulation in non-small cell lung cancer

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Abstract. Insulin receptor substrate-1 (IRS-1) is an adaptor protein for insulin-like growth factor (IGF) signaling and it is presumed associated with cancer development, progression or clinical outcome of patients harboring solid tumors. Therefore, we investigated by immunohistochemistry, the expression of IRS-1 in the tumor tissues from 94 patients who were diagnosed as stage I non-small cell lung cancer (NSCLC) and had undergone a curative lung resection. The relationships between its intratumoral expression and various clinical parameters were explored. IRS-1 is consistently expressed in the cytoplasm of intrapulmonary bronchial and bronchiolar epithelial cells comprising normal appearing adjacent lung tissues. Forty-one (43.6%) of 94 specimens showed loss of IRS-1 expression. In a subset analysis, IRS-1 was more frequently lost in stage IB than in IA tumors (50.0 vs. 22.7%, $p=0.024$, χ^2 test), which was reflected by the facts that tumors which showed down-regulation of IRS-1 had larger area than those with IRS-1 expression (18.1 vs. 12.1 cm², $p=0.044$, t-test). Down-regulation of IRS-1 is more frequently observed in squamous cell carcinoma than other cell type lung cancer ($p=0.002$, χ^2 test) and its expression was not affected by histological grade of differentiation. Comparing pack-years (P.Y.) between groups of smokers whose tumor expressed IRS-1 and those that did not, smokers whose tumor showed loss of IRS-1 expression had higher P.Y. than those whose tumor did express IRS-1 (39.2±23.67 vs. 25.6±26.61 P.Y., $p=0.034$, t-test). Intratumoral expression of IRS-1 did not influence disease-free survival, disease-specific survival or overall survival of stage I NSCLC patients, whose median follow-up duration is 7.5 years (95% CI; 7.21-7.86 years).

These results suggest that loss of IRS-1 might rather be an early event in NSCLC development than a prognostic factor and that it is more strongly related with squamous cell carcinoma and with smoking.

Introduction

Despite years of intense efforts to control lung cancer mortality with surgical resection, chemotherapy and radiotherapy, it remains the most common cause of cancer death worldwide. This dismal outlook clearly indicates urgent need for elucidation of the mechanisms of lung cancer carcinogenesis, as well as new approaches for prevention and treatment.

Insulin-like growth factors (IGFs) are synthesized by almost all tissues and regulate cell proliferation, differentiation and apoptosis, all important attributes to tumorigenesis (1). Several studies on cancer risks have suggested that high levels of circulating IGF-1 are associated with increased risk of solid cancers including non-small cell lung cancer (NSCLC) (2-5). Binding of IGF-1 to its receptor (IGF-1R) results in receptor autophosphorylation and subsequent phosphorylation of Shc and insulin receptor substrate (IRS)-1, -2, -3 and -4. (6).

IRS-1, which is critical to IGF signaling, belongs to the IRS family of adaptor molecules and is tyrosine-phosphorylated in response to IGF-1, insulin and other cytokines (7,8). Phosphorylation of Tyr⁶⁰⁸ and Tyr⁶²⁸ of IRS-1 (using rat IRS-1 numbering) generates the major docking sites for phosphatidylinositol 3-kinase (PI3K) and activates multiple signaling pathways, including PI3K, mitogen-activated protein kinase (MAPK) and Akt (9). Activation of these signaling pathways induces multifunctional biologic actions of IGFs, including cell growth, differentiation and survival. On the other hand, serine/threonine phosphorylation of 50 potential sites of IRS-1 is regarded to oppose the biologic activity of tyrosine phosphorylation (8). Besides phosphorylation of IRS-1, chronic exposure to IGFs, mannitol, okadaic acids, high glucose and hyperinsulinemia decreased the cellular level of IRS-1, which blunted response to apoptotic signals (10). Aberrant expression of IRS-1 has been associated with the pathogenesis of many diseases, including diabetes and cancer of the breast, pancreas, prostate, and liver (11-14). By extension, a delicate balance between 'positive' IRS-1 tyrosine

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Table I. Analysis of the patients with stage 1 NSCLC according to the expression status of IRS-1.

	IRS-1 expression in tumor tissue		P-value
	Down-regulated (n=41)	Expressed (n=53)	
Age ^b (years)	63.3±8.93	60.6±10.59	0.205
Gender			
Male	33	39	0.433
Female	8	14	
Smoking status			
Smoker; pack-year ^b	24; 39.2±23.67	26; 25.6±26.61	0.077; 0.034 ^a
Non-smoker	4	13	
Unknown	13	14	
Pathology			
Adenocarcinoma	12	35	0.002 ^a
Squamous cell carcinoma	26	17	
Other NSCLC cell type	3	1	
Histological grade			
Well differentiated	13	17	0.749
Moderately differentiated	10	19	
Poorly differentiated	9	12	
Unclassified	9	5	
TNM stage			
T1N0M0	5	17	0.024 ^a
T2N0M0	36	36	
Tumor area ^b (cm ²)	18.1±14.55	12.1±13.76	0.044 ^a
Mean survival time, (months), (95% CI)			
Disease-free	98.6 (79.5-117.7)	93.6 (80.8-106.4)	0.422
Disease-specific	111.4 (93.8-129.0)	102.5 (91.1-113.8)	0.562
Overall	109.0 (91.3-126.7)	97.6 (86.0-109.1)	0.829

^ap-value <0.05. ^bMean ± standard deviation.

phosphorylation and 'negative' serine/threonine phosphorylation with decreased IRS-1 could regulate the functions of IRS-1, and shifts of this equilibrium could lead to pathological situations (8).

Current understandings on the IGF system suggest that post-receptor signaling pathways are important in elucidating the involvement of the IGF system in lung cancer development. Previous research suggest that IRS-1 expression in NSCLC tissue might be associated with development and/ or clinical outcome of lung cancer (15,16). Consequently, we evaluated IRS-1 expression in 94 stage I NSCLC tissue samples and investigated it with clinical and pathological findings.

Materials and methods

Study population. Ninety-four specimens were obtained from patients diagnosed as stage I NSCLC between 1993 and

2004. All patients had undergone a curative surgical removal of a primary lesion at the time of diagnosis and had not received either radiotherapy or chemotherapy before or after surgery until disease recurred. Histopathological diagnosis and classification of tumors was based on criteria of the World Health Organization (WHO). The post-surgical, pathological tumor-node-metastasis (TNM) stage of each malignancy was determined according to the guidelines of the American Joint Committee on Cancer. Details of clinical and pathological characteristics are presented in Table I.

Immunohistochemical staining (IHC) of IRS-1. IHC was performed using the LABS[®] 2 system (Dako Corp., Carpinteria, CA, USA) according to the manufacturer's instructions. Briefly, paraffinized sections were dewaxed in xylene baths and rehydrated using a graded alcohol series. Following deparaffinization, sections were immersed in methanol for

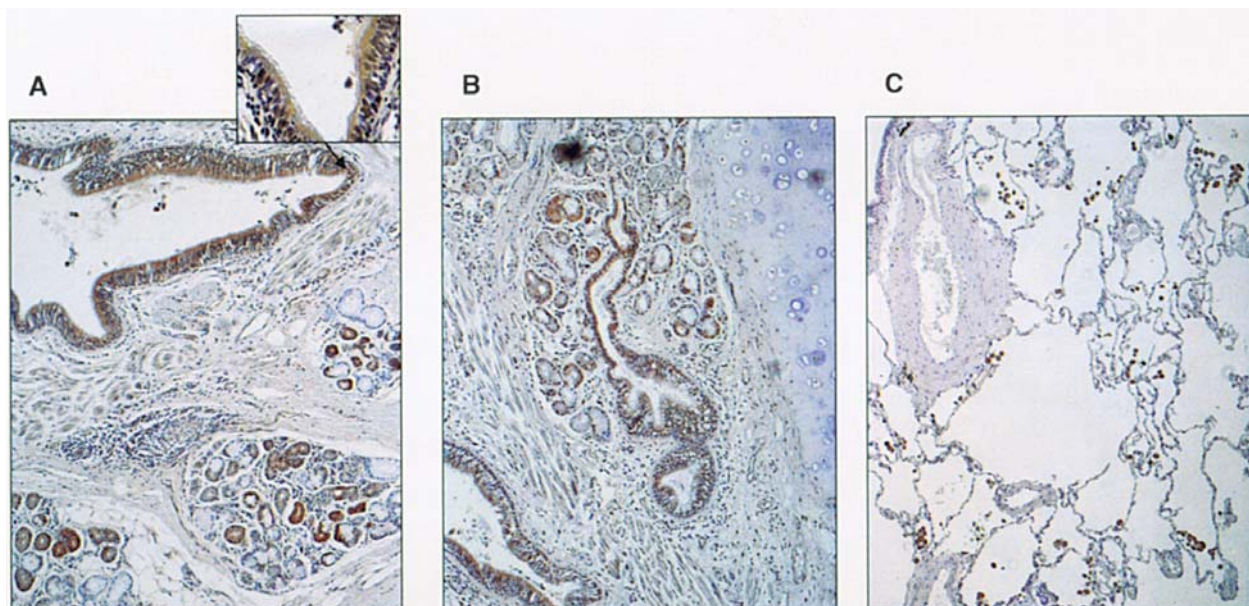


Figure 1. IRS-1 expression in normal-appearing adjacent lung tissue. Cytoplasm of epithelial cells comprising intrapulmonary bronchi (A) and bronchioles (B) showed strong and constant IRS-1 expression. Nuclei of those cells were also frequently stained with anti-IRS-1 antibody (A, inset). Other cells comprising lung tissue, including type 1 and 2 alveolar cells, vascular endothelial cells were not stained by anti-IRS-1 antibody (C). (Magnification x200; inset, x400).

5 min to block endogenous peroxidase activity and then incubated for 10 min with primary anti-IRS-1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in antibody diluent (Dako Corp.) at 1:100 dilution. Sections were incubated for another 10 min with biotinylated linker and processed using avidin-biotin IHC techniques. Each experiment included positive and negative controls. 3,3'-diaminobenzidine (DAB) was used as a chromogen in conjunction with the Liquid DAB substrate kit (Novacastra, UK). All slides were examined by light microscopy and classified by pathologists who were blinded to the relevant clinical information. Cancer cells in at least four fields were counted at x200 magnification. IRS-1 immuno-reactivity was classified by the proportion of positive cells; 0, <5% positive cells; 1+, 5-30% positive cells; 2+, 30-50% positive cells; 3+, >50% positive cells. Intensity of IRS-1 expression was scored as negative to weak (0), moderate (1) or strong (2). The score is the sum of intensity and percentage of positive cells and a score ≤ 1 was applied as a cut-off for loss of IRS-1 expression.

Statistical analysis. Survival data of all patients were obtained from the Korea National Statistical Office on June 17, 2005. In univariate analyses, independent sample t- and χ^2 tests were used for continuous and categorical variables, respectively. The Kaplan-Meier estimator was used to compute the survival probability as a function of time. The log-rank test was used to compare survival times between patient groups. Overall, disease-specific and disease-free survival times were also analyzed. All statistical tests were two-tailed, and a p-value <0.05 was considered statistically significant.

Results

Expression of IRS-1 in adjacent normal-appearing lung tissue. To get a positive control for IHC, authors explored IRS-1

expression in NSCLC cell lines by Western blot. NCI-H157, -H358, -H596, -H1299, -H1792 and -A549 cell lines exhibited strong IRS-1 expression and, therefore, a cell block of -H1299 was included as an external positive control in each experiment with Ki-67 staining (data not shown). IRS-1 expression was prominent in the cytoplasm of normal-appearing bronchial and bronchiolar epithelial cells and localized in the perinuclear area. The intensity of IRS-1 staining in the cytoplasm was strong, granular and distinct (Fig. 1A and B). IRS-1 expression in the cytoplasm of bronchial and bronchiolar epithelial cells was constant and used as a positive internal control in this study. Nuclei of bronchial and bronchiolar epithelial cells also reacted with anti-IRS-1 antibody frequently, however, its immunoreactivity was neither homogeneous nor uniform among bronchi and bronchioles (Fig 1A, inset). Type I and II pneumocytes, endothelial cells and other cells comprising lung tissue did not react with anti-IRS-1 antibody except the cytoplasm of submucosal glandular cells (Fig. 1C).

IRS-1 expression in NSCLC tissue and clinical and pathological parameters associated with its expression. Forty-seven (50.0%) out of the 94 NSCLCs were adenocarcinoma, 43 (45.7%) squamous cell carcinomas, and 4 (4.3%) other histologic type NSCLCs. IHC analysis of NSCLC tissue samples revealed that IRS-1 expression was lost in 41 (43.6%) NSCLC samples, using a 5% cut-off (Fig. 2). One of the conspicuous findings in this study is that IRS-1 is more frequently down-regulated in squamous cell carcinoma than in other histologic subtypes ($p=0.002$, χ^2 -test) (Fig. 2A and B). We were able to classify 80 tissue samples for histologic grade of differentiation. Thirteen (43.3%) out of 30 well, 10 (34.5%) out of 29 moderately, and 9 (42.9%) out of 21 poorly differentiated NSCLC tissue lost IRS-1 expression and found that IRS-1 expression was not influenced by grade of differentiation. In a subgroup analysis among subtypes of adenocarcinoma, no

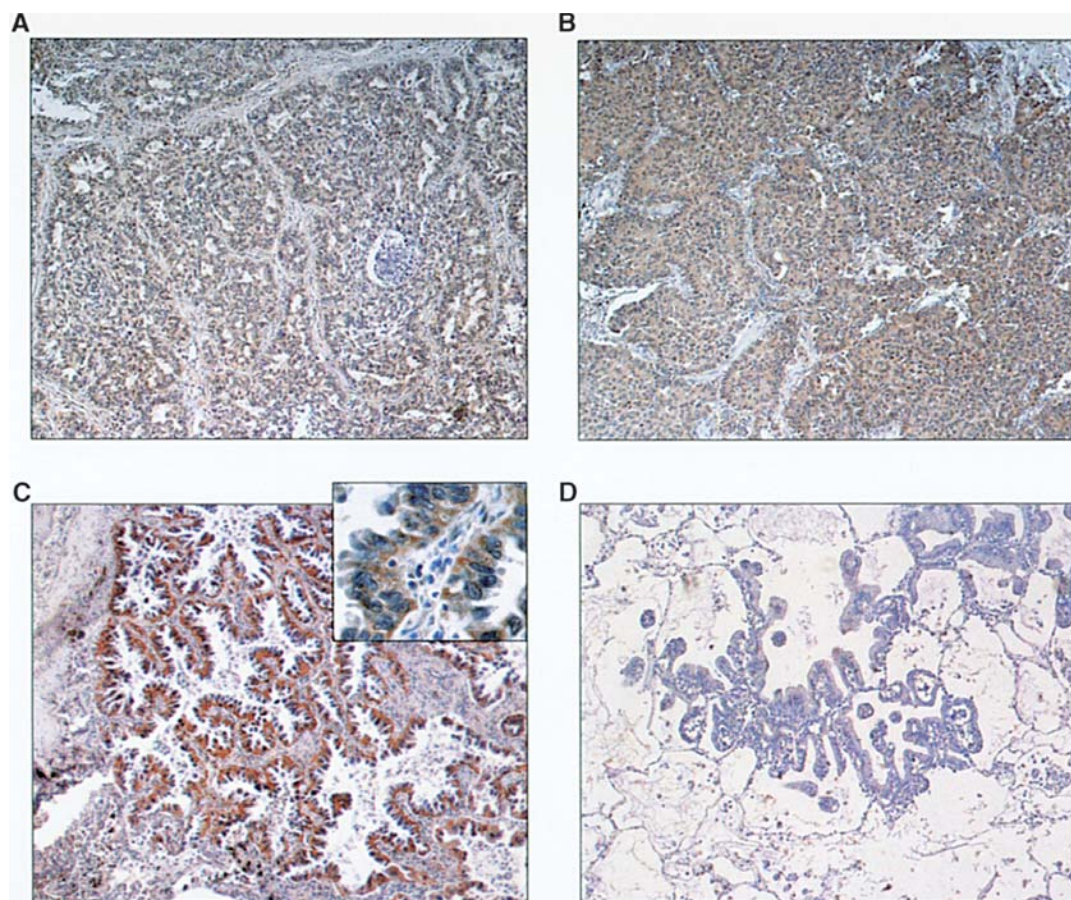


Figure 2. IRS-1 expression in NSCLC tissue. Adenocarcinoma (A) and squamous cell carcinoma (B) of lung showed strong immunoreactivity with IRS-1. A bronchioloalveolar carcinoma has strong IRS-1 expression (C; inset, x400), whereas other do not (D). (Magnification x200).

differences were observed between bronchioloalveolar carcinomas and other subtypes of adenocarcinoma (Fig. 2C and D).

Patient age and gender did not differ between groups whose tumors did and did not exhibit IRS-1 expression. NSCLC tissues from smokers more frequently lost IRS-1 expression than those from non-smokers but this did not reach statistical significance ($p=0.077$, χ^2 test). Comparing pack-years (P.Y.) between groups of smokers whose tumor expressed IRS-1 and those that did not, smokers whose tumor lost IRS-1 expression had higher P.Y. than those whose tumor did express IRS-1 (39.2 ± 23.67 vs. 25.6 ± 26.61 P.Y., $p=0.034$, t -test). IRS-1 was more frequently down-regulated in the tumor tissues with TNM IB than in those with TNM IA status (50.0 vs. 22.7% , $p=0.024$, χ^2 test). This was reflected by the finding that tumors positive for IRS-1 expression were on average larger than IRS-1-negative tumors (18.1 ± 14.55 vs. 12.1 ± 13.76 cm², $p=0.044$, t -test). Table I summarizes the associations between expression status of IRS-1 and clinical and pathological parameters.

Survival analysis. The median follow-up duration of the patients who were alive at the time of analysis was 7.5 years (95% CI; 7.21-7.86 years). Twenty-nine (30.9%) out of 94 patients died during the follow-up period and 25 (26.6%) died of cancer-related events. The 5-year survival probability of the entire patient population was 70.1%, which is similar to a previous report from a large-scale study (17). Intratumoral

IRS-1 expression status did not make differences in disease-free, disease-specific, and overall survival of NSCLC patients (Table I, Fig. 3). Subgroup analyses classified according to pathologic diagnosis, grade of differentiation or TNM stages also showed that IRS-1 expression status did not influence disease-free, disease-specific, and overall survival of NSCLC patients (data not shown).

Discussion

IGF signaling cascades may be involved in lung cancer development. It is supported by several large scale epidemiologic studies which had shown that high IGF-1 and low IGFBP-3 levels are independent risk factors of lung cancer (2,18). Because IRS-1 is a major mediator of IGF signaling, we inferred that IRS-1 may play a specific role in NSCLC carcinogenesis or progression. To explore the role of IRS-1 in NSCLC, authors confined study objects to a homogeneous group diagnosed as stage I NSCLC and who underwent curative surgery at the time of diagnosis.

IRS-1 was strongly and consistently expressed in the cytoplasm of intrapulmonary bronchial and bronchiolar epithelial cells comprising normal-appearing lung tissues. Previous studies using normal-appearing breast tissues showed that IRS-1 was strongly to moderately expressed in the cytoplasm of ductal and acinal epithelial cells and that nuclei were sometimes immunoreacted with IRS-1 (16). Our study showed that

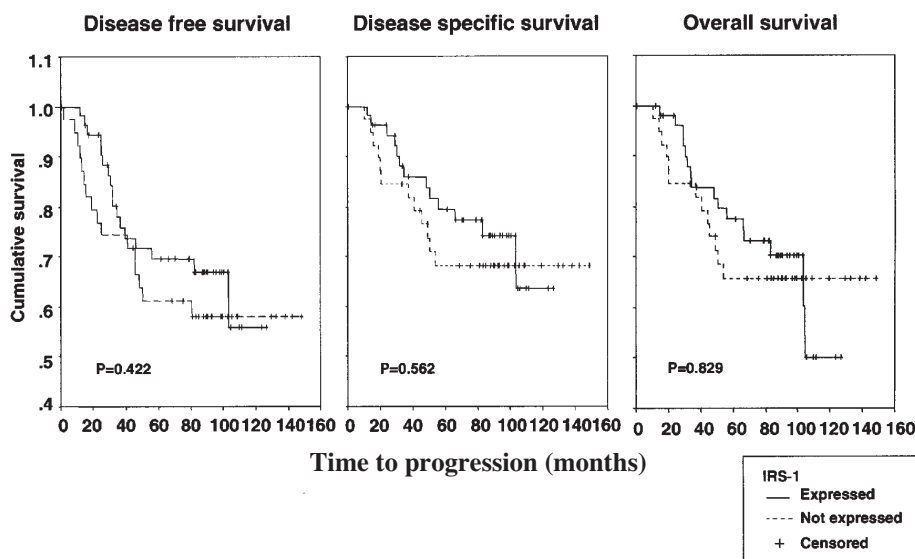


Figure 3. Survival analysis of stage I NSCLC patients according to IRS-1 expression. The intratumoral expression of IRS-1 did not influence disease-free survival (A), disease-specific survival (B) or overall survival (C) of NSCLC patients.

IRS-1 expression was more frequently lost in the NSCLC tissues with larger diameters (T2 tumors) than the smaller ones. The underlying mechanisms for down-regulation of IRS-1 still remain unknown but down-regulation of IRS-1 was also observed in the progression of breast cancer and experimental hepatocarcinogenesis, which indicates that this represents a more general disturbance in signaling during cancer development (15,16,19). Cellular level of IRS-1 is post-translationally modulated by multiple pharmacological reagents, such as retinoic acid derivatives and ubiquitin proteasome pathway modulators (20). Although physiologic level of circulating IGF-1 protects against degradation of IRS-1 by various stimuli, prolonged exposure to high concentrations of IGF-1, high osmolarity, high glucose, and okadaic acid also accelerate IRS-1 degradation (10,21). Elucidating the mechanisms of IRS-1 down-regulation and finding the materials that regulate expression and/or function of IRS-1 in NSCLC *in vivo* may be of help for modulation of cancer progression.

There are contradictory reports on the relationship between cancer differentiation and IRS-1 expression. Schnarr *et al* indicated that well (grade 1) and moderately differentiated (grade 2) ductal carcinoma of breast has moderate to high IRS-1 expression than poorly differentiated ones (16). However, Koda *et al* reported that IRS-1 is more frequently expressed in the breast cancer tissues with poorly differentiated (grade 3) histology and with lymph node involvement (22). Careful attention must be paid to the fact that Koda *et al* did not clarify IRS-1 expression in normal adjacent breast tissues and did not compare intratumoral IRS-1 expression with that in normal adjacent tissues. Our study revealed that 43% of well, 34% of moderately and 42% of poorly differentiated NSCLC tissues showed loss of IRS-1 expression and suggest that histologic grade of differentiation did not influence IRS-1 expression in NSCLC tissue.

IRS-1 was more frequently lost in squamous cell carcinoma of the lung than adenocarcinoma. The underlying mechanisms for more frequent loss in squamous cell carcinoma is unknown

but it may be partially supported by earlier studies investigating the consequences of IGF and IGF receptor dysfunction in lung cancer. Pavelic *et al* suggested a correlation between increased expression for IGF-1 and IGF-1R that has protective property against IRS-1 degradation, as well as decreased apoptosis in adenocarcinomas (23).

The significance of IRS-1 expression for prognosis in cancers has been a matter of debates because of conflicting results (16). High IRS-1 level, which is determined by ELISA or immunoblotting, were associated with early breast cancer recurrence (24,25). We confined the study to the homogeneous cases, and showed that intratumoral IRS-1 expression did not influence the prognosis of patients when analyzed in terms of disease-free, disease-specific and overall survival. Taken together, intratumoral down-regulation of IRS-1 is suggested to be a molecular event related to the early development of NSCLC rather than a prognostic factor. Down-regulation of IRS-1 might be of help for classification of NSCLC because it shows a more frequent loss in squamous cell carcinoma than other cell types. More comprehensive studies of the mechanisms that induce loss of IRS-1 expression in NSCLC are necessary for the elucidation of the role of IRS-1 in lung carcinogenesis. Insights gained from this basic study may lead to novel clinical research targeted at prevention and therapy of NSCLC.

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