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Frequency and clinicopathologic correlates of *KRAS* amplification in non-small cell lung carcinoma

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ABSTRACT

Background: Characterization of the non-small cell lung cancer (NSCLC) genome has suggested that KRAS amplification is one of the commonest molecular abnormalities in NSCLC. However, the prevalence and clinicopathologic significance of KRAS amplification, and its relationship with *KRAS* activating mutations have not been well-defined. The purpose of this study was to establish the prevalence of *KRAS* amplification in two separate, large NSCLC cohorts, to define the clinicopathologic features of *KRAS*-amplified NSCLC in a single uniformly treated cohort, and to investigate the interplay between *KRAS* amplification and *KRAS* mutation.
Methods: Fluorescence in situ hybridization was utilized to detect *KRAS* amplification on tissue microarrays constructed from a Swiss cohort of 538 NSCLCs and a series of 402 patients with NSCLC treated in a single institution in New York. DNA sequencing to detect *KRAS* codon 12 activating mutations was performed on a subset of tumors. Amplification and mutation status were compared with patient baseline characteristics, tumor characteristics, and overall- and disease-free survival.
Results: The prevalence of *KRAS* amplification was 13.7% in the Swiss cohort and 15.1% in the New York cohort. Among adenocarcinomas, *KRAS* amplification was associated with larger (mean size 2.8 ± 1.8 cm vs. 2.1 ± 1.3 cm, $p = 0.003$), less well-differentiated tumors (18% vs. 42%, $p = 0.004$) that were more likely to be invasive (95% vs. 77%, $p = 0.004$) and to exhibit angiolymphatic invasion (24% vs. 12%, $p = 0.04$). These differences were statistically significant within the subset of adenocarcinomas harboring activating *KRAS* mutations, suggesting a synergistic relationship between amplification and mutation. No significant association between *KRAS* amplification and nodal metastasis or survival was seen.
Conclusions: KRAS amplification is a common molecular alteration in NSCLC, characterizing ~15% of tumors. This alteration is associated with indicators of local aggressiveness, and may act synergistically with *KRAS* mutations to promote tumor progression.

1. Introduction

In a wide variety of human cancers, activating mutations in RAS oncogenes contribute to tumorigenesis by promoting cellular proliferation in the absence of normal stimuli for growth and replication. The oncogenic effects of RAS proteins are manifested when specific codons (12, 13 and 61) are mutated such that their gene products are no longer susceptible to counter-regulatory signals, rendering them constitutively active. The myriad downstream effects of dysregulated RAS activity facilitate unchecked cell division and the accumulation of additional oncogenic mutations. In lung cancer, this chain of events is well characterized, and KRAS mutations are associated with a discrete subset of tumors defined by a strong association with tobacco exposure, locally advanced disease, limited prognosis, and poor response rates to tyrosine kinase inhibitors [1].

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By contrast, the role of RAS gene amplification as an oncogenic mechanism remains relatively undefined. A number of recent studies employing high-resolution techniques to map human tumor genomes have consistently identified copy number gains at 12p12.1, including the KRAS gene, in a wide spectrum of malignancies, including lung cancer [2–8]. Using fluorescence in situ hybridization, we previously confirmed the presence of KRAS amplification in a minority of non-small cell lung carcinomas (NSCLCs), and demonstrated an association between KRAS amplification and increased expression of the p21 gene product [3–11,22]. In a similar report, Modrek et al., detected copy number gains of KRAS in ~17% of NSCLCs and found that amplification was more likely in patients with activating KRAS mutations [12]. These authors established a strong correlation between KRAS amplification and increased KRAS mRNA levels.

On the basis of these findings, evidence for a clear relationship among KRAS gene amplification, activating mutation and protein expression has emerged. However, the clinicopathologic significance of these findings remains unknown. In order to better define the relationship between KRAS amplification and activating mutation, and to investigate the clinical relevance of KRAS amplification, we determined its frequency in two large cohorts of NSCLC, utilizing fluorescence in situ hybridization. We then correlated KRAS amplification status with the presence of KRAS activating mutations, standard clinicopathologic features, and outcome data.

2. Methods

2.1. Patients and tumor samples

The University of Zurich cohort is a population based cohort of 538 patients with NSCLC who underwent surgery between January 1993 and December 2002 at the University Hospital Zurich and surrounding referral hospitals, and has been previously described [13,14]. Histologic tumor types included in the cohort were adenocarcinoma, squamous cell carcinoma, and adenosquamous carcinoma, while large cell carcinoma, neuroendocrine carcinoma, sarcomatoid carcinoma, and metastases from primaries other than lung were excluded. Surgical lung specimens were processed according to the guidelines of the Swiss Society of Pathology. The New York Hospital-Weill Cornell Medical Center cohort comprises 402 surgically resected NSCLCs in a single institution over the period 1992–2007, including 302 consecutive and uniformly treated tumors between 2005 and 2007. Patient and tumor characteristics were obtained via chart review. Final clinical and pathologic staging and follow up information were derived from a prospectively maintained thoracic surgical registry; the median length of follow-up among survivors was 2.9 years. In both cohorts, formalin-fixed, paraffin-embedded tissue cores of 0.6 mm diameter (2–3 per tumor) were incorporated into tissue microarrays. Non-neoplastic control lung tissue was incorporated into each microarray. The study was approved independently by the Institutional Ethical Review Boards of the University Hospital Zurich and the New York Presbyterian Hospital/Weill Cornell Medical Center.

2.2. Fluorescence in situ hybridization

To assess for KRAS amplification, the Biotin-14dCTP labeled BAC clone CTD-2174F1 (eventually conjugated to produce a red signal) was used for the KRAS gene locus (chr 12p12.1) and the Digoxigenin-dUTP labeled BAC clone RP11-482D24 (eventually conjugated to produce a green signal) was used as reference probe spanning a stable region in lung adeno- and squamous cell-carcinomas (chr12q23.3). The BAC clones were obtained from the BACPAC Resource Center, Children's Hospital Oakland Research Institute (Oakland, CA) and also from Invitrogen (Carlsbad, CA). Before tissue analysis, correct chromosomal probe localization was confirmed on metaphase spreads of normal peripheral lymphocytes. After deparaffinization, tissue samples were pretreated in 100 mM Tris and 50 mM EDTA solution at 92 °C, pH 7.0; buffer for 15 min and washed with PBS at room temperature. For protein digestion Digest-All III (dilution 1:2) was put on the slides for 20 min. After washing the slides in PBS again, they were dehydrated in ethanol (70%, 85%, 95%, 100%, 2 min each) at room temperature. The probes were denaturated at 73 °C for 5 min and immediately placed on ice. The slides and probes were co-denatured at 94 °C for 3 min and hybridized overnight at 37 °C. Post-hybridization washing was done with 2× SSC at 75 °C for 5 min. Samples were rinsed three times for 2 min with 0.5× SSC buffer at room temperature. Blocking was performed in a dark moist chamber with CAS-Block containing 10% goat serum for 10 min. The fluorescence detection was carried out using streptavidin-Alexa-594 conjugates (dilution 1:200) and anti-digoxigenin-FITC (dilution 1:200). Samples were washed three times with 0.5× SSC buffer as above before mounting with 4′,6-diamidin-2′-phenylindoldihydrochlorid (DAPI). Samples were analyzed under an 63× oil immersion objective using a fluorescence microscope (Zeiss, Jena, Germany) equipped with appropriate filters, a charge-coupled device camera and the FISH imaging and capturing software Metafer 4 (Metasystems, Altlussheim, Germany). A minimum of 100 nuclei per tumor was deemed necessary for accurate assessment, and cases with fewer than 100 assessable nuclei were excluded. The assessment of the KRAS amplification status was made semi-quantitatively by comparing the target (KRAS) and reference probe signals. Low-level amplification was defined if there were more than three additional target (KRAS) signals relative to the number of reference probe signals in at least 30% of tumor cells. Cases with more than ten additional target signals relative to the number of reference probe signals were defined as having high level KRAS amplification. The evaluation was independently performed by two evaluators (ACS and TW). In rare cases with heterogeneity in the degree of amplification, for statistical purposes the status corresponding to the region with the highest degree of amplification was recorded.

2.3. DNA sequencing

DNA was extracted from formalin-fixed, paraffin-embedded tumor tissue using the phenol/chloroform method. PCR primer sequences were selected to amplify sequences surrounding codon 12 in exon 1, which are involved in ~85% of activating KRAS mutations. The primer sequences, obtained from TIB Molbiol (Berlin, Germany) were 5′-AGGCCTGCTGAAAATGACTG-3′ (forward), 5′-AGAATGGTCCTGCACCAGTAA-3′ (reverse) for exon 1. PCR products were amplified using the cycling conditions of 2 min at 95 °C, 50 cycles each with 45 s at 95 °C, 1 min and 15 s at 58 °C, 1 min at 72 °C and a final elongation step of 7 min at 72 °C. These products were then analyzed with the Light cycler 640 using primers specific for the mutation. The results were exhibited as peaks specific for a particular base pair.

2.4. Statistical analysis

Correlation between KRAS amplification status and clinicopathologic variables was assessed using Student's t test for continuous variables and chi square or Fisher's exact test as appropriate for categorical variables. Overall and disease-free survival were assessed using the Kaplan–Meier method, with differences among subgroups analyzed using the log-rank test (SPSS Statistics version 17.0, SPSS Inc., Chicago, IL, USA). For all tests, $p < 0.05$ was considered statistically significant.