

Chromosomal instability is more frequent in metastasized than in non-metastasized pulmonary carcinoids but is not a reliable predictor of metastatic potential

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Abbreviations: CGH, comparative genomic hybridization; CIN, chromosomal instability; MIN, microsatellite instability

Abstract

Pulmonary carcinoids are infrequent neoplasms of the lung that normally display a less aggressive biological behavior compared to small cell and non-small cell lung cancers. Approximately 15-25% of carcinoids, in particular atypical carcinoids, show lymph node metastasis and have a worse prognosis than their non-metastasized counterparts. To date, there is no morphological or molecular marker that may help to differentiate between carcinoids that metastasize and carcinoids of identical differentiation that show only local tumor growth. In this study, we analyzed 7 metastasized and 10 non-metastasized pulmonary carcinoids for chromosomal and microsatellite instability in order to determine whether microsatellite instability or chromosomal imbalances are associated with metastasis. Due to the rare occurrence of metastasized carcinoids we compared our results of chromosomal instability with the hitherto published comparative genomic hybridization (CGH) profiles of pulmonary carcinoids, for which information about the absence or presence of metastasis was available. While microsatellite instability was not detected we found chromosomal instability as a common event in pulmonary carcinoids

with an increase of frequency and extent of chromosomal alterations in atypical and metastasized carcinoids. These findings are in accordance with the collected and herein compiled data of previous studies and indicate increasing numbers of chromosomal imbalances to play a role in the sequential process of tumor development and metastasis.

Keywords: biological markers; carcinoid tumor; comparative genomic hybridization; lung neoplasms; microsatellite instability; neoplasm metastasis

Introduction

Carcinoids (well-differentiated endocrine tumors) are neoplasms derived from the disseminated neuroendocrine system. About 10% of all human carcinoid tumors arise in the lungs (Godwin, 1975). Pulmonary carcinoids are currently classified as typical carcinoids (TC; low grade; fewer than 2 mitoses per 2 mm², lack of necrosis) or atypical carcinoids (ATC; intermediate grade; 2-10 mitoses per 2 mm² and/or foci of necrosis). Although TC and ATC show subtle differences in histomorphology and although ATC tend to show a more aggressive biological behavior, both carcinoid subtypes have the potential to metastasize (Beasley *et al.*, 2000). In a large series of 124 pulmonary carcinoids, 19 tumors had regional lymph node metastases and 12 had distant metastases (McCaughan *et al.*, 1985). The 5- and 10-year disease-free survival rate in 19 patients who had regional lymphatic metastases were 74% and 53%, compared with 96% and 84% in those without lymph node metastases. Therefore it was concluded, that among other factors such as tumor size, patient's prognosis is strongly influenced by the status of the regional lymph nodes (McCaughan *et al.*, 1985).

Chromosomal and microsatellite instability (CIN and MIN) are two distinct molecular mechanisms that cause DNA mutations and that underlie the pathogenesis of many epithelial tumors. Tools for the detection of gross chromosomal aberrations in a tumor with CIN on the one hand and DNA mismatch mutations on a molecular level on the other hand are comparative genomic hybridization

(CGH) and PCR-based microsatellite instability (MSI) analyses, respectively. Both methods are applicable in routine, paraffin-embedded tumor material. To date, only few data are available concerning CGH and MSI analyses of pulmonary carcinoids (Hurr *et al.*, 1996; Ullmann *et al.*, 1998, 2002; Zhao *et al.*, 2000; Johnen *et al.*, 2003; Vageli *et al.*, 2006). In these studies, chromosomal imbalances were identified both in ATC and TC, but none of these studies has investigated potential differences in chromosomal aberrations between metastasized and non-metastasized pulmonary carcinoids. The detection of specific genetic changes associated with more aggressive, metastasizing behavior and the identification of genes regulating the metastatic phenotype of pulmonary carcinoids would be helpful for both the planning of treatment strategies and patient follow-up.

We have analyzed microsatellite and chromosomal alterations in a collective of 17 metastasized and non-metastasized pulmonary carcinoids by CGH and MSI analyses. In addition, all published CGH analyses of pulmonary carcinoids were reviewed with regard to the specifications of the different alterations between metastasized and non-metastasized specimens. Our data show an evidently higher frequency of chromosomal instability in metastasized carcinoids and indicate that most carcinoids acquire a metastatic potential through accumulation of chromosomal alterations on the background of CIN.

Results

The results of our CGH analyses are summarized in Table 1. Chromosomal imbalances were found in 9 specimens (~53%) while 8 tumors (~47%) had no chromosomal imbalances detectable by conventional CGH. Two specimens showed a deletion of chromosome 11, the most common reported finding in pulmonary carcinoids. Interestingly, there were two metastasized tumors without any detectable chromosomal aberrations (Table 1) and one non-metastasized tumor with aberrations on 5 different loci (Table 1, Case 16).

Data from the meta-analysis of hitherto published CGH analyses (Walch *et al.*, 1998; Zhao *et al.*, 2000; Ullmann *et al.*, 2002), including our own findings from pulmonary carcinoids, are summarized in Figure 1. In general, ATC harbour more chromosomal aberrations than TC. The frequency of CIN is evidently increased in metastasized versus non-metastasized carcinoids (gains 71% vs 51%, losses 76% vs 51%, cases with no chromosomal alterations 14% vs 31%, respectively). It is interesting to note that in contrast to non-metastasized tumors, no obvious difference in CIN frequency is found between metastasized TC and ATC (89% in TC versus 83% in ATC). Increase of chromosomal losses was found comparing TC to ATC and comparing non-metastasized to metastasized carcinoids (Figure 1). In detail, there are more gains in metastasized carcinoids compared

Table 1. CGH analyses of the investigated pulmonary carcinoids.

Case	Diagnosis	TNM*	Gains	Losses
1	ATC	pT3, pN1, M0	+9, +16p	-3p, -16q
2	ATC	pT2, pN0, M0	-	-
3	ATC	pT2, pN0, M0	+18	-16
4	ATC	pT2, pN0, M0	-	-
5	ATC	pT2, pN1, M0	-	-
6	ATC	pT2, pN2, M0	+4, +8, +9, +16, +20	-11, -14q
7	ATC	pT3, pN1, M0	-	-6q, -13q
8	ATC	pT2, pN1, M0	-	-22
9	ATC	pT1, pN0, M0	-	-1p, -2q
10	ATC	pT1, pN0, M0	-	-
11	TC	pT1, pN0, M0	-	-
12	TC	pT1, pN2, M0	+7	-3, -11q
13	TC	pT1, pN0, M0	+8	-
14	TC	pT4, pN1, M0	-	-
15	TC	pT2, pN0, M0	-	-
16	TC	pT1, pN0, M0	+9	-1q, -3p, -3q, -10p
17	TC	pT1, pN0, M0	-	-

*Following the current WHO classification for lung cancer (Brambilla *et al.*, 2001). ATC, atypical carcinoid; TC, typical carcinoid.

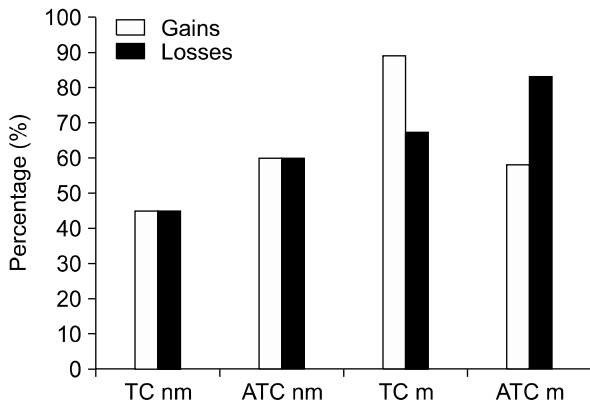


Figure 1. Summarized meta-analysis data of CIN frequency from all available CGH studies on metastasized and non-metastasized pulmonary carcinoids. TC, typical carcinoids; ATC, atypical carcinoids; nm, non-metastasized; m, metastasized.

to non-metastasized carcinoids on chromosomes 5q, 16 and 17 (Walch *et al.*, 1998; Zhao *et al.*, 2000; Ullmann *et al.*, 2002). Although only revealed by one of the studies (Ullmann *et al.*, 2002) a notable finding is the loss of chromosome 18 material exclusively in metastasized carcinoids.

MSI analyses were performed in 16 out of 17 carcinoids. We could not identify any specimen with MSI in either Cat-25, Bat-25 or Bat-26. Therefore the tumors were scored as microsatellite stable (MSS).

Discussion

Previous studies of genetic alterations in pulmonary carcinoids have focused on the differences between ATC and TC, differences between a few primary tumors and corresponding metastases, or the classification of pulmonary carcinoids within the group of neuroendocrine neoplasms of the lungs (Ullmann *et al.*, 1998, 2002; Walch *et al.*, 1998; Zhao *et al.*, 2000; Johnen *et al.*, 2003). Genetic differences between metastasized and non-metastasized pulmonary carcinoids have not been specifically addressed to date. Since lymph node or distant metastases of pulmonary carcinoids significantly influence patient survival (McCaughan *et al.*, 1985), more information on the genetic background of tumor dissemination may be helpful to develop novel treatment and follow-up strategies. Therefore, we aimed to shed more light on differences in genetic aberrations between metastasized and non-metastasized pulmonary carcinoids. A recent study (Arnold *et al.*, 2008) has demonstrated microsatellite instability (MSI) in some malignant neuroendocrine tumors of the intestines. In

order to investigate the role of MSI in the development of pulmonary carcinoids in general and metastasis in particular we analysed the herein presented carcinoids for MSI and could exclude a significant role of this molecular mechanism for the pathogenesis and progression of carcinoids. On the other hand, our data show that CIN is frequently involved in tumor development. Moreover, the increase of CIN in metastasizing carcinoids indicates that CIN also influences tumor progression. In metastasized TC, the increase of CIN similarly affects losses and gains of chromosomal material. In metastasized ATC, the number of gains remains stable, while a significant increase of genomic losses is seen (Figure 1), indicating that loss of function mutations are involved in the process of tumor dissemination. For pancreatic endocrine neoplasms, similar observations concerning an increase of CIN in metastasized versus non-metastasized tumors were previously reported (Jonkers *et al.*, 2005)

Our data suggest a model of carcinoid development and progression in which mitotic infidelity causes CIN at an early stage. During tumor progression, especially chromosomal losses inactivate the functions of tumor suppressor genes and promote metastatic behaviour. The fact that two metastasized carcinoids in our study lacked CIN, however, indicates that a pathway different from the acquisition of numerous gross chromosomal alterations exists. It is likely that in these tumors chromosomal imbalances beyond the sensitivity threshold of conventional CGH or small intragenic mutations in tumor suppressor genes have occurred.

Knowledge of a specific genetic alteration associated with metastasis would largely add to our understanding of the different biological behavior of pulmonary carcinoids and would enable to assess the risk of tumor dissemination by investigating biopsy material prior to surgery. We were unable to detect a chromosomal alteration with a satisfying sensitivity and specificity for metastasis in our cohort of cases. It is of note, however, that Zhao *et al.* (2000) described deletions at chromosome 18q in 4 of 7 metastasized carcinoids while no 18q loss was identified in non metastasized pulmonary carcinoids. Deletions of chromosome 18q are common in a variety of human malignancies. They have been described e.g. in gastrointestinal neuroendocrine tumors (carcinoids) (Zhao *et al.*, 2000) and non-small cell lung cancer (Pan *et al.*, 2005). Deletions of chromosome 18q are generally associated with advanced tumor stage in NSCLC (Pan *et al.*, 2005) and have most interestingly been found in about 58% of metastasized gastroin-

testinal carcinoids, whereas no losses of chromosome 18q were present in non-metastasized gastrointestinal carcinoids (Zhao *et al.*, 2000). However, our study and two previous studies (Walch *et al.*, 1998; Ullmann *et al.*, 2002) failed to identify chromosome 18 deletions in metastasized pulmonary carcinoids, indicating a poor sensitivity of this alteration concerning the prediction of metastatic potential.

In conclusion, we herein show that chromosomal instability is increased in atypical compared to typical and metastasized compared to non-metastasized pulmonary carcinoids. Our data suggest that chromosomal losses, in particular in ATC, lead to the inactivation of tumor suppressor genes and contribute to the metastatic capabilities of endocrine neoplasms.

Methods

Tumor samples and preparation

Samples from 10 ATC and 7 TC were investigated in this study. 5 of the ATC and 2 of the TC had regional lymph node metastases (Table 1). All tumors were surgically removed including extensive lymph node dissection in the Department of Thoracic Surgery, Heidelberg, Germany, resulting in a minimum of 11 and a maximum of 35 lymph nodes. Diagnostic radiology was performed to detect distant metastases in all cases. According to both, clinical and radiological findings, none of the patients had distant metastases. All specimens were routinely processed in the Institute for Pathology, Heidelberg University Hospital, Germany. All diagnoses were confirmed by at least two experienced pathologists and tumor typing was performed according to the current lung cancer classification from the World Health Organization (WHO) (Brambilla *et al.*, 2001). Postoperative follow-up (maximum 6 years) was evaluated in all cases and all patients were alive and free of tumor recurrence at the time of this study.

DNA extraction

DNA used for CGH and MSI analyses was isolated from paraffin-embedded samples with a tumor content of at least 80% according to the corresponding histological slide. Genomic DNA was exclusively isolated from tumor primaries as reported previously in detail (Blaker *et al.*, 2002).

Comparative genomic hybridization (CGH)

CGH was performed on all 17 pulmonary carcinoids as previously described in detail (Isola *et al.*, 1994). In brief, tumor DNA was biotinylated and normal control DNA was extracted from healthy human placenta tissue and labeled with digoxigenin. After hybridization of the labeled DNA on metaphase spreads (CGH target slides, Abbot Molecular, Illinois) at 37°C for 48 h, the slides were incubated with

fluorescence-labeled anti-digoxigenin and anti-biotin antibodies. Metaphase spreads from each specimen were analyzed and photodocumented using an Axiovert S 100 microscope (Zeiss, Germany), a CCD camera, and hardware and software as supplied by Metasystems (Altlusheim, Germany). Fluorescence ratios were determined using both fixed cut-off values (0.8 for losses, 1.25 for gains) and cut-off values with a two-fold SD. Chromosomal abnormalities detected with both approaches were scored. Centromeric and telomeric chromosomal areas were excluded.

Microsatellite instability (MSI) analysis

Microsatellite marker Cat-25 (Findeisen *et al.*, 2005) and Bethesda markers Bat-25 and Bat-26 were analyzed. Primer sequences were chosen as published previously (Findeisen *et al.*, 2005). Microsatellite-PCR reactions were performed on microtitre plates with 9 µl reaction mixture (1 µl 10× buffer (Peqlab), 1.5 mM MgCl₂, 0.5 mM dNTP, 0.5 U Taq-Polymerase (Solis Biodyne), 0.75 µM specific primers, ad 9 µl H₂O) and 1 µl DNA using a Thermo Hybrid Omn-E thermal cycler (Ulm, Germany). PCR products were diluted in equal volumes of formamide buffer and denatured at 95°C for 1 min. Finally, PCR products were separated on 6% polyacrylamide, 8 M urea gels and visualized by silver staining.

Compilation of all available data concerning metastasized carcinoids

All published CGH analyses of pulmonary carcinoids that were clearly classifiable as "metastasized", both hematogenous and lymphogenous, or "non-metastasized" were included (Walch *et al.*, 1998; Zhao *et al.*, 2000; Ullmann *et al.*, 2002). Cases without the required information were excluded, yielding a total of 21 metastasized carcinoids (12 ATC, 9TC) and 54 non-metastasized carcinoids (23 ATC, 31 TC).

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