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Analysis of centrosome overduplication in correlation to cell division errors in high-risk human papillomavirus (HPV)-associated anal neoplasms

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Abstract

High-risk HPV-associated anal neoplasms are difficult to treat and biomarkers of malignant progression are needed. A hallmark of carcinogenic progression is genomic instability, which is frequently associated with cell division errors and aneuploidy. The HPV-16 E7 oncoprotein has been previously shown to rapidly induce centriole and centrosome overduplication and to cooperate with HPV-16 E6 in the induction of abnormal multipolar mitoses. Based on this function, it has been suggested that HPV-16 E7 may act as a driving force for chromosomal instability. However, a detailed analysis of centrosome overduplication in primary HPV-associated neoplasms has not been performed so far. Here, we determined the frequency of centrosome overduplication in HPV-associated anal lesions using a recently identified marker for mature maternal centrioles, Cep170. We detected centrosome overduplication in a small but significant fraction of cells. Remarkably, centrosome overduplication, but not aberrant centrosome numbers *per se* or centrosome accumulation, correlated significantly with the presence of cell division errors. In addition, our experiments revealed that in particular pseudo-bipolar mitoses may play a role in the propagation of chromosomal instability in high-risk HPV-associated tumors. These results provide new insights into the role of centrosome aberrations in cell division errors and encourage further studies on centrosome overduplication as a predictive biomarker of malignant progression in HPV-associated anal lesions.

Keywords: Anal carcinoma; HPV-16; Centrosome aberrations; Cell division errors; Cep170

Introduction

Infection with high-risk HPV types such as HPV-16 is intimately associated with squamous cell carcinomas and pre-cancerous lesions of the anogenital tract (Palefsky, 1995; Palefsky et al., 1991). Men who have sex with men (MSM), in particular those who are seropositive for the human immunodeficiency virus (HIV), but also HIV-positive women, have a high risk for anal HPV infection and the development of high-grade anal intraepithelial lesions (AIN) and anal squamous cell carcinomas (Palefsky, 1998). Such lesions are difficult to treat and surgical therapy is frequently associated with significant post-operative morbidity (Abbasakoor and Boulos, 2005; Berry et al., 2004). Hence, the identification of novel biomarkers to predict malignant progression of AIN would help to improve patient management and quality of life (Palefsky, 2006).

High-grade intraepithelial anal neoplasia has been proposed to represent a pre-cancerous lesion similar to HPV-associated cervical intraepithelial neoplasia (CIN). In CIN, malignant progression has been found to be associated with increasing aneuploidy and centrosome aberrations (Heselmeyer et al., 1997; Kashyap and Das, 1998; Monsonego et al., 1997; Skyldberg et al., 2001; Steinbeck, 1997). Centrosomes are small cytoplasmic organelles that function as microtubule organizing centers in most animal and human cells. They consist of a pair of centrioles, short barrel-shaped microtubule cylinders, which are embedded in pericentriolar material (PCM). During mitosis, centrosomes are involved in the organization and orientation of a bipolar mitotic spindle. Prior to mitosis, the single centrosome duplicates in synchrony with the cell division

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cycle. In this process, a pre-existing mother centriole serves as a nucleation center for normally only one newly synthesized daughter centriole (Bettencourt-Dias and Glover, 2007; Nigg, 2007; Sluder, 2004; Tsou and Stearns, 2006). The regulatory mechanisms that limit daughter centriole synthesis are poorly characterized but previous studies have shown that certain oncogenic stimuli, including the HPV-16 E7 oncoprotein (see also below), can rapidly disrupt this process (Duensing et al., 2007).

Centrosome aberrations are readily detectable in tumor samples (Lingle et al., 1998), but their use as cancer biomarkers has been hampered by the fact that it was not possible to distinguish between centrosome anomalies that potentially drive chromosomal instability and those that merely arise as a side effect of unrelated cellular insults such as abortive mitoses or incomplete cytokinesis (Duensing and Munger, 2001; Nigg, 2002). This distinction, however, is critical because centrosome aberrations may have fundamentally different consequences dependent on the cellular context in which they occur (Duensing, 2005).

High-risk HPVs encode two oncoproteins, E6 and E7, which function during the viral life cycle by promoting viral genome replication in differentiating keratinocytes. Whereas the HPV E6 oncoprotein inactivates the p53 tumor suppressor, the HPV E7 oncoprotein binds and degrades the retinoblastoma tumor suppressor protein (pRB) as well as the related proteins p107 and p130. HPV E7 is a multifunctional protein and several additional targets have been identified, including the cyclin-dependent kinase (CDK) inhibitors $p21^{Cip1}$ and $p27^{Kip1}$, cyclins, histone deacetylases (HDACs) and several transcription regulators (Longworth and Laimins, 2004; Munger and Howley, 2002; zur Hausen, 1996).

The HPV-16 E6 and E7 oncoproteins not only stimulate abnormal centrosome numbers, but they have also been instrumental in dissecting distinct mechanisms by which centrosome amplification may arise. The HPV-16 E7 oncoprotein was found to rapidly trigger an excessive production of daughter centrioles thereby rendering cells prone to multipolar mitoses and chromosomal aberrations in a subsequent cell division (Duensing et al., 2001, 2000). Abnormal centrosome numbers were detected before cells became genomically unstable indicating that they may function as a driving force for chromosomal instability (Duensing et al., 2001). Moreover, it was recently discovered that HPV-16 E7 can promote the concurrent formation of more than one daughter centriole at a single mother, a phenotype that is normally not seen in proliferating cells but that can occur during ciliogenesis (Duensing et al., 2007). In contrast, the HPV-16 E6 oncoprotein was found to provoke an accumulation of centrosomes in cells that were frequently growth-arrested and hence unlikely to generate viable daughter cells and propagate chromosomal instability. Nonetheless, high-risk HPV E6 cooperates with the HPV E7 oncoprotein in the induction of abnormal mitoses and aneuploidy, most likely by relaxing p53 responses (Duensing et al., 2001).

The two basic mechanisms of centrosome amplification, centrosome overduplication and centrosome accumulation, can be discriminated by immunostaining for a novel centrosomal protein, Cep170 (Duensing et al., 2006; Guarguaglini et al., 2005). Cep170 specifically labels mature mother centrioles and therefore allows the distinction between a genuine overduplication of centrosomes in the presence of a single mature mother and centrosome accumulation that usually leads to multiple mature mother centrioles (Guarguaglini et al., 2005). The question whether centrosome overduplication exists *in vivo*, however, has not been addressed in detail.

Here, we analyzed a total of 44 benign, pre-malignant or malignant HPV-associated anal lesions by double-immunofluorescence microscopy for γ-tubulin and Cep170 in order to ascertain the frequency of centrosome overduplication. We detected centrosome overduplication in a small but significant number of cells and found that it correlates with the presence of cell division errors. Remarkably, aberrant centrosome numbers *per se* or centrosome accumulation did not correlate significantly with the presence of cell division errors. We provide evidence that in particular pseudo-bipolar cell divisions may contribute to the propagation of chromosomal instability in human tumors whereas multipolar mitoses may not. Collectively, our results suggest that primary centrosome overduplication increases the risk for cell division errors in high-risk HPV-associated anal neoplasms and encourage further studies into the potential use of centrosome overduplication as a surrogate marker for chromosomal instability and carcinogenic progression.

Results

Centrosome overduplication in high-risk HPV-associated anal lesions

Formalin-fixed, paraffin-embedded specimens from a total of 44 anal lesions were analyzed (Table 1). All anal squamous

Table 1
Diagnosis, HPV status and summary of centrosome and mitotic aberrations

Diagnosis	*n*=	HPV-16/-18/-31/-33 positive	HPV-6/-11 positive	Mean % of cells with aberrant centrosome numbers	Mean % of cells with centrosome overduplication	Mean % of cells with centrosome accumulation	Mean % of abnormal metaphases per HPF [a]	Mean % of abnormal ana-/telophases per HPF
SCC	14	14	1	5.8%	0.7%	5.2%	0.4%	0.04%
HSIL	13	13	4	2.5%	0.2%	2.2%	0.3%	0.03%
LSIL	6	6	6	4.7%	0.1%	4.6%	0.02%	0.02%
Control [b]	11	nd [c]	nd	1%	0.03%	0.9%	0%	0%

SCC, squamous cell carcinoma; HSIL high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion.
[a] High-power field.
[b] Squamous epithelial tissue from hemorrhoidal excision specimens.
[c] Not done.