CEBPD amplification and overexpression in urothelial carcinoma: a driver of tumor metastasis indicating adverse prognosis

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ABSTRACT

The molecular aberrations responsible for the progression of urothelial carcinoma (UC) remain largely obscure. To search candidate driver oncogenes in UC, we performed array-based genomic hybridization (aCGH) on 40 UBUC samples. Amplification of 8q11.21 was preferentially identified in patients who developed disease-specific death (53.8%) and distal metastasis (50.0%) but was barely detected in non-eventful cases (3.7% and 0%, respectively). In order to quantify the expression of candidate genes harbored in 8q11.21, laser-capture microdissection coupled with RT-PCR was performed on 32 of the 40 cases submitted to aCGH. With this, we identified *CEBPD* mRNA expression as most significantly associated with gains of 8q11.21, suggesting amplification-driven expression. By performing *CEBPD*-specific FISH and immunohistochemistry on 295 UBUCs, we confirmed *CEBPD* amplification (21.3%) and overexpression (29.8%) were strongly related to each other ($p<0.001$). Moreover, both were associated with adverse clinicopathologic features and worse outcomes. Furthermore, the clinical significance of CEBPD expression was also confirmed in an independent cohort comprised of 340 UCs from the upper urinary tract. Interestingly, *CEBPD* knockdown suppressed cell proliferation, migration and, most significantly, cell invasion ability in UC cells. The latter phenotype is attributed to downregulation of MMP2 as identified by RT^2 Profiler PCR array. Moreover, expression of CEBPD significantly enhanced MMP2 expression and transcriptional activation by directly

binding to its promoter region, as confirmed by promoter reporter assay and chromatin immunoprecipitation assay. Conclusively, *CEBPD* amplification is a mechanism driving increased mRNA and protein expression that confers aggressiveness in UC through MMP2-mediated cell invasiveness.

INTRODUCTION

Urothelial carcinoma (UC), the most common malignancy of the genitourinary tract, originates from the urothelium of the urinary bladder (UBUC) and upper urinary tract (UTUC). Interestingly, UBUC and UTUC share common morphologic, etiologic, and pathological features. Moreover, the gene expression profiles of UCs from different locations are very similar [1]. There is a strong possibility that all UCs, regardless of location, may share a common molecular pathway in carcinogenesis. In past years, investigations focusing on genomic aberrations in UC revealed that complex cytogenetic aberration is characteristic of aggressive behavior. Defining these alterations may help us to understand the genetic hallmarks of tumor progression and help identify new molecular signatures that can be used for better diagnosis, prognostication and the development of more effective therapeutic strategies.

Array-based genomic hybridization is essential in searching for the key chromosomal regions harboring critical genes. In UC, chromosomal gains in 1q, 3p, 6p, 8q, 10p, 17q, and 20q are frequently identified [2, 3]. The gain of chromosome 8q24 harboring *MYC* in particular has been suggested to be associated with UC progression. However, in the literature, the prognostic implications of gains involving different regions of chromosome 8q have been inconsistent, and the derived candidate oncogenes remain largely undefined for UC. To search for candidate oncogenes relevant to tumor progression, we performed aCGH analysis of 40 UBUCs (Table-S1) and identified chromosome 8q as the most significant differentially gained region in UCs (up to 75%) associated with adverse outcomes. Of the whole chromosome 8q, we focused special attention on the gain in 8q11.21, since it was most relevant to the development of distal metastasis and also one of the top-ranking altered regions associated with the development of disease-specific death. Given recurrent gains spanning its DNA locus and significantly increased mRNA expression in UCs with poor outcomes, we specifically selected CCAAT/enhancer binding protein delta (*CEBPD*) at 8q11.21 to evaluate its biological and clinical relevance using cell lines and independent samples.

CCAAT/enhancer binding protein delta (*CEBPD*) is a transcription factor implicated in physiological processes such as cell differentiation, metabolism, inflammation, growth arrest and cell death [4, 5], yet its role in cancer remains much debated. Initially, studies suggested CEBPD acts as a tumor suppressor in leukemia [6-8], prostate cancer [9] and hepatocellular carcinoma [10]. Intriguingly, recent work using a *Cebpd* knockout mouse model to explore mammary tumorigenesis indicated that CEBPD may promote tumor metastasis [11]. One study reported that CEBPD expression level correlates with development of chemotherapy resistance in patients with UC [12]. Based on these seemingly contradictory results, CEBPD could be associated with and contribute to either a better or worse prognosis, depending on the tumor type or cell of origin. To confirm its true function in specific kinds of cancer requires further investigation.

Here we are the first to report that gene amplification is a mechanism that drives CEBPD overexpression in UC, and that its expression correlates with poor clinical prognosis. We confirmed that CEBPD enhances cell growth in UC cell lines by promoting G1-S cell cycle transition. We also showed that CEBPD enhances motility and invasiveness of UC cells via direct promoter binding and active transcription of matrix metalloproteinase-2 (MMP2). These findings reinforce the oncogenic function of CEBPD in UC and contribute to clarifying the molecular mechanisms of how CEBPD promotes tumor metastasis.

RESULTS

Recurrent 8q11.21 amplicon spanned *KIAA0146, CEBPD, PRKDC, MCM4,* and *UBE2V2* was preferentially identified in UBUC with poor outcomes

Varying degrees of chromosomal imbalances were detected in all UBUC samples subjected to aCGH profiling. Using Nexus Copy Number™ software, we identified more recurrent regions of gains than deletions across the whole genome in UBUCs. Consistent with the previous literature [13], the most common chromosomal aberrations (Figure-S1) identified in at least half of samples were -9p, +8q, and -5q, which were detected in 60%, 55%, and 50% of the samples, respectively. Other common recurrent alterations with varying extent of involvement included +1q, -2q, -3p, +3q, -4q, +5p, -5q, -6q, +7p, -7q, -8p, -9q, +10p, -10q, -11p, +11q, -13q, -17p, +17q, +18p, -18q, +19q, +20, +22q; we identified these in 20-50% of samples. Computerized by Nexus Copy Number™ software, the recurrent chromosomal aberrations are summarized in Table-S2. Of these, the gains involving 8q showed most significant preference to UBUCs with poor outcomes, exhibiting differential frequencies of 54.4% and 70.8% when comparing patients