Human papillomavirus

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Unexpected heterogeneity in oropharyngeal squamous cell tumors

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A study uses single-cell RNA sequencing to profile human papillomavirus (HPV)-positive and -negative oropharyngeal squamous cell carcinoma, revealing considerable diversity within and between tumors. Within HPV-positive tumors, subsets of malignant cells are found with undetectable HPV expression and decreased HPV-related phenotypes, which may influence prognosis and response to therapy.

Oropharyngeal squamous cell carcinoma (OPSCC) is a subset of head and neck cancer that is rapidly growing in incidence, and up to 90% of OPSCCs are now commonly associated with human papillomavirus (HPV) infection[1,2]. HPV is a sexually transmitted DNA virus that is implicated in a variety of cancers. The HPV oncogenic proteins E6 and E7 inhibit the tumor suppressors TP53 and RB1, respectively[3–5], increasing genetic instability and aberrant proliferation of infected epithelial cells. In this issue of *Nature Genetics*, Puram and Mints et al.[6] report provocative discoveries concerning the heterogeneity that exists within and between HPV-positive and -negative OPSCC.

Using single-cell RNA sequencing (scRNA-seq), the team profiled 16 treatment-naive OPSCC tumor samples. These cells were correspondingly clustered by their expression profiles into discrete categories based on cell type (for example, epithelial, non-epithelial and so on), genetic clones, cellular states and HPV expression patterns. Twelve of the tumors were HPV positive and four HPV negative, as shown via standard p16 staining. Transcripts of HPV16 (the most common high-risk genotype) were identified in 11 of the tumors that were clinically defined as HPV positive and were absent in tumors defined as HPV negative. Patterns and levels of expression of specific HPV16 genes varied between and within individual tumors.

The team further characterized epithelial cells as malignant or non-malignant by inferring copy-number aberrations (CNAs) — estimating copy numbers of chromosomal loci by averaging normalized expression levels of 100 adjacent genes and comparing against a reference set of fibroblasts and endothelial cells[7]. All but two of the tumors showed evidence of multiple malignant subclones, with one tumor possessing as many as six distinct subclones. Many of these CNAs were characteristic of squamous cell carcinoma, including loss of 3p and gain of 3q (ref. 8).

Using an algorithmic approach known as non-negative matrix factorization, the team was able to define eight groups of recurrent gene expression programs, deemed 'meta-programs'. These meta-programs included cell cycle (G1/S and G2/M phase), stress and hypoxia responses, oxidative phosphorylation, interferon response, hybrid, partial epithelial-to-mensenchymal transition (EMT) and an epithelial senescence-associated (EpiSen) program, corresponding to different cellular states. Similar meta-programs or modules have been reported across a variety of cancers, including glioblastoma, pancreatic ductal adenocarcinoma and other head and neck cancers[7,9,10].

Aside from the G2/M meta-program, these meta-programs were found to differ significantly in expression between HPV-positive and -negative tumors. In addition, within the HPV-positive tumors, there were notable differences in meta-program expression between malignant cells that had detectable HPV reads (deemed *HPVon*) and

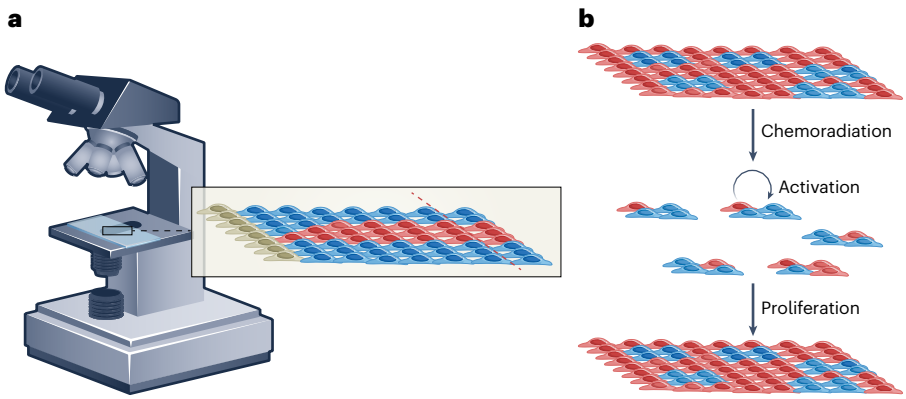

Fig. 1 | Clinically undetected heterogeneity in *HPVoff* and *HPVon* cells. **a**, A negative surgical margin (red dashed line) beyond clinically positive p16-stained cells (brown). Beyond the margin exists a proposed malignant subclone (red) that has evolved undetectably to immunohistochemical staining but is potentially detectable by molecular techniques. **b**, *HPVoff* cells (blue) gaining *HPVon* (red) functionality and proliferating after chemoradiation.

those without detectable HPV reads (deemed *HPVoff*). The team postulated that these differences in expression may represent unique cellular states associated with the genetic or epigenetic repression of HPV genes. For example, the G1/S meta-program was enriched in *HPVon* cells relative to *HPVoff* and HPV-negative cells, while the EpiSen meta-program was enriched in *HPV*off cells relative to *HPVon* cells. The relative enrichment of the G1/S meta-program in *HPVon* cells may reflect aberrant activation by HPV E7, while the relative enrichment of EpiSen in *HPVoff* cells may reflect the reduced activity of HPV E6. These results suggest that within a given HPV-positive tumor, there may exist a subset of cells that have an undetectable level of HPV-derived mRNA, yielding phenotypes that at least partially resemble HPV-negative cells.

One might speculate that the phenomenon of *HPVoff* cells may represent a mechanism protective of HPV—with parallels to drug-tolerant persistent cells[11]. Indeed, the authors[6] report that *HPVoff* cells had a decreased response to standard cytotoxic cancer treatment (cisplatin) and increased invasion in vitro. Additionally, individuals with HPV-positive tumors with a larger proportion of *HPVoff* cells trend toward worse disease-free survival. Essentially, cells in the *HPVon* state, which possess increased cell-cycle activity, are more amenable to chemotherapy and radiation; whereas *HPVoff* cells maintain a senescence-like state, evading treatment and possibly later restoring a proliferative *HPVon* state. Further experiments conducted with HPV-positive cell lines suggest a possible epigenetic mechanism, as treatment of these cell lines with EZH2 and DNA methyltransferase inhibitors (tazemetostat or decitabine) led to a significant reduction in E6 and E7 expression.

Moreover, malignant clones exist within normal tissues and the ever-present immune system of the host. Results of a recent study based on single-cell sequencing in lung cancer suggest the existence of larger communities or 'neighborhoods' of cells that are distinctly organized within the tumor microenvironment. These included neighborhoods of varying B and T cell populations within the tumor tissue. So-called pan-immune hotspots and B-cell-enriched neighborhoods carried a more favorable prognosis. It is tempting to speculate that the *HPVoff* populations in OPSCC tumors may also arise as a response to the activated immune cells in their neighborhood[12]. Indeed, natural HPV infection results in a weak and type-specific immune response, and there remains a need to develop effective therapeutic vaccines to better eradicate HPV-positive tumors[13].

The authors[6] also profiled and compared epithelial cells in tissue from surgical margins that had been deemed histologically negative in three cases (Fig. 1a). As expected, most of these cells were classified as non-malignant. However, in one negative margin sample, malignant cells were identified via CNA analysis. A subset of these cells was found to express HPV genes and unique CNAs, in addition to possessing all the CNAs shared across subclones from the tumor. Earlier molecular work based on detection of cells with a tumor-specific p53 mutation demonstrated the existence of malignant clones beyond the histological edge of the tumor[14]. The presence of promoter hypermethylation or scRNA-seq could be deployed in the clinical setting to verify the presence or absence of residual malignant cells at surgical margins[15,16].

The work reported here reveals the protean nature of HPV-positive and -negative OPSCC tumors, highlighting the challenges of developing effective targeted therapies for these tumors. Although OPSCCs caused by HPV tend to be associated with a more favorable prognosis, a subset of these tumors responds poorly to treatment and recurs[17] (Fig. 1b). These findings advance our understanding of HPV in OPSCC tumors and open important avenues to explore, in an effort to further improve patient outcomes.

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Competing interests

The authors declare no competing interests.