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Intratumoral heterogeneity promotes disease progression and altered angiogenesis in EGFR-mutant NSCLC tumor xenografts.

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Lung cancer is the leading cause of cancer deaths worldwide, with NSCLC being the most common histological subtype. The majority of NSCLC patients are diagnosed with an advanced disease and the development of effective systemic therapies are still necessary. Although activating epidermal growth factor receptor (EGFR) kinase domain mutations sensitizes NSCLC to EGFR tyrosine kinase inhibitors (TKIs), many NSCLC patients succumb to acquired resistance, mediated by secondary EGFR mutations (T790M) or the activation of epithelial-to-mesenchymal transition (EMT), among other mechanisms. Besides, one of the hallmarks of cancer is intratumor heterogeneity. We have previously demonstrated that genetic and epigenetic heterogeneity within EGFR-mutant NSCLC cells lines gives rise to divergent resistance mechanisms (emergence of T790M subpopulation or activation of EMT) in response to TKI treatment.

In this work, we have developed i*n vivo* admix models composed of both epithelial and mesenchymal NSCLC cells inoculated in nude mice to study intratumoral heterogeneity and elucidate therapeutic responses. While NSCLC cell with acquired EGFR TKI resistance and EMT phenotype did not exhibit any growth advantage in vivo, a 50% epithelial/mesenchymal admix provided significant growth advantage and TKI resistance *in vivo*. Interestingly, short-term *in vitro* co-culture of epithelial and mesenchymal cells did not provide any proliferative advantage. These results led us to hypothesize that the epithelial/mesenchymal admix helps to create a tumor-host niche that is suitable for EGFR TKI resistance. To this end, we utilized the Luminex multiplex assay system to quantify secreted growth factors, cytokines, and chemokines. We discovered that epithelial EGFR TKI sensitive cells secrete predominantly the proangiogenic factor VEGF-A, while mesenchymal EGFR TKI resistant cells do not, secreting significant amounts of endothelin-1 (EDN1). Ectopic overexpression of EDN1 in EGFR TKI sensitive cells decreased VEGF secretion and more importantly, conferred significant resistance to gefitinib in vivo, but not *in vitro*, suggesting that TKI resistance may be related to the alteration of these angiogenic factors in the tumor microenvironment.

EDN1 secreting mesenchymal cells exhibited remarkable growth retardation in vivo but not in vitro. However, it was unclear if EDN1 modifies host vasculature to reduce not only nutrient but also drug supply to the tumors. We performed CD31 staining to test if the presence of EDN1-overexpressing tumors decreases the number and size of blood vessels. We found a significant decrease in microvessel density seen in both mesenchymal or 50% epithelial/mesenchymal admix tumors, compared to epithelial EGFR TKI sensitive cells. These results support the notion that there is an angiogenic switch characterized by increased secretion of EDN1 in heterogeneous NSCLC tumors enriched in mesenchymal cells, a condition that eventually compromises intratumoral vascularity, a new mechanism contributing to TKI resistance *in vivo*. Besides, these evidences suggest that the therapeutic rationale of combining EGFR TKIs with anti-VEGF drugs in the treatment of EGFR-mutant NSCLC should be revised.

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