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Intra-tumoral Heterogeneity in Osteosarcoma and its Therapeutic Implications

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Background. Osteosarcoma (OS) is a virulent form of bone cancer that primarily affects children. The long-term survival rates for OS have remained stagnant over the past 30 years, and ~40% of the children who develop osteosarcoma will die of it. The disparity in prognoses among OS patients likely stems from the heterogeneous nature of the cells that comprise these tumors, which vary widely with regard to differentiation, proliferation rate, tumorigenicity and metastatic ability.

Toward the development of treatment paradigms with greater efficacy, we have been investigating the nature of intra-tumoral heterogeneity in OS, working to identify the mechanisms that support malignancy in certain subpopulations of cells, and those by which it is naturally silenced in others. Investigations into intra-tumoral heterogeneity have primarily used differential expression of cell-surface antigens as a means to identify divergent cell populations in a variety of solid tumors. For each malignancy, certain markers have been found that enable enrichment for cells with increased tumorigenic activity but, no single antigen or antigen combination has been found to provide a definitive tumorigenic signature. Thus, it has been impossible to cleanly segregate the tumorigenic and non-tumorigenic populations within tumors for comparative analysis.

In previous work we developed a method that enables identification of tumor initiating cells from osteosarcoma xenografts based on their transcriptional activity rather than surface antigen expression. We hypothesized that tumor cells that were the most extensively “de-programmed” would possess the greatest malignant potential and thus be capable of activating promoters for proteins normally restricted to embryonic stem (ES) and primordial germ (PG) cells. To test this hypothesis we used an exogenous full-length human Oct4 promoter-driven GFP reporter construct to identify tumor initiating cells (TIC) from primary osteosarcoma biopsies. Following xenotransplantation, GFP+ cells showed a greater than 100-fold enrichment for tumorigenic activity relative to GFP- cells. Moreover, GFP+ cells were highly clonogenic in vitro and were found to divide almost exclusively by symmetric cell division; however, subsequent transplantation led to tumors that were composed of both GFP+ and GFP- cells. Given the increased tumorigenicity of the GFP+ fraction, these observations indicate a spontaneous/naturally-occurring loss of the malignant phenotype during tumor growth.

Purpose. In the present study we have adapted this experimental paradigm by incorporating global transcriptional profiling and bioinformatics analyses in order to identify the specific molecular networks associated with the tumorigenic and non-tumorigenic cell populations isolated from two histologically distinct OS xenografts. 1) Is loss of tumorigenicity associated with stochastic or deterministic changes in global gene transcription? 2) Are the clusters of DE genes enriched for specific biological pathways related to malignancy? 3) Are the transcription

patterns reflective of protein expression and/or activation status? 4) Are the transcriptional signatures predictive of cell function?

Methods. cDNA microarray analysis was used to generate global transcriptional profiles of GFP+ and GFP- sub-populations harvested from tumor xenografts generated by serial transplantation of GFP+ cells from two histologically distinct primary OS cell lines. Statistical analysis and hierarchal clustering algorithms were used to identify sets of differentially expressed (DE) genes associated with each phenotype. Biological relevance was extracted through gene ontology (GO) analysis of each gene set using the Functional Annotation Enrichment and Clustering tools provided by DAVID. Western analyses were used to validate the transcriptional data with respect to expression and/or activation status of the encoded proteins and functional assays were used to assess its predictive value regarding the biological potential/behavior of each cell type.

Results. 1) Differential expression and supervised hierarchal cluster analyses of global gene expression indicate loss of tumorigenic potential is a deterministic process. 2) GO analysis of the DE genes revealed the transcriptional signature of tumorigenic cells is enriched for biologic processes driving proliferation and genetic instability whereas non-tumorigenic cells are highly enriched for genes and pathways regulating cellular response to stress. 3) Western analyses prove the transcriptional data significantly correlated to relative expression levels and/or activation status of encoded proteins. 4) Flow cytometric analysis of cell cycle progression showed that the tumorigenic cell fraction contained twice the proportion of cells in G2 and M phases of the cell cycle compared to the non-tumorigenic cell fraction of the same xenograft.

Conclusions. Our findings suggest that dysregulation the of G2/M and SA checkpoints enables the tumor-initiating cell to override these rate-limiting steps to proliferate at an accelerated rate. Conversely they indicate that extrinsic and intrinsic chronic/severe cellular stress, from oxygen and nutrient deprivation, or irreparable genetic damage directs the biology of the initiating cell away from proliferation, to survival and adaptation, effectively reversing the malignant phenotype.