Molecular or Metabolic Reprograming: What Triggers Tumor Subtypes?

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Conflict of interest

A. Sadanandam is entitled to a share of royalty received by the licensor for a patent entitled "Colorectal cancer classification with differential prognosis and personalized therapeutic responses" – Patent number PCT/IB2013/060416.

Abstract

Tumor heterogeneity is reflected and influenced by genetic, epigenetic and metabolic differences in cancer cells and their interactions with a complex microenvironment. This heterogeneity has resulted in the stratification of tumors into subtypes, mainly based on cancer-specific genomic or transcriptomic profiles. Subtyping can lead to biomarker identification for personalized diagnosis and therapy, but stratification alone does not explain the origins of tumor heterogeneity. Heterogeneity has traditionally been thought to arise from distinct mutations/aberrations in "driver" oncogenes. However, certain subtypes appear to be the result of adaptation to the disrupted microenvironment caused by abnormal tumor vasculature triggering metabolic switches. Moreover, heterogeneity persists despite the predominance of single oncogenic driver mutations, perhaps due to second metabolic or genetic "hits". In certain cancer types, existing subtypes have metabolic and transcriptomic phenotypes that are reminiscent of normal differentiated cells, whereas others reflect the phenotypes of stem or mesenchymal cells. The cell-of-origin may, therefore, play a role in tumor heterogeneity. In this mini-review, we focus on how cancer cell-specific heterogeneity is driven by different

genetic or metabolic factors alone or in combination using specific cancers to illustrate these concepts.

Introduction

Tumor heterogeneity refers to variations in genotype and phenotype between different tumors (inter-tumoral heterogeneity) or cells in a single tumor (intra-tumoral heterogeneity). The existence of inter-tumoral heterogeneity is well established and illustrated by the gene expression profiles used to stratify multiple cancer types, including, but not limited to, leukemias, glioblastoma, breast, pancreatic and colorectal tumors into their molecular subtypes (1-6). However, the true extent of intra-tumoral molecular heterogeneity is only just being elucidated, in part due to the recent exploitation of high-throughput genomic analyses of multiple biopsies from individual tumors or by the isolation and analysis of single cells (7). In general, heterogeneity in cancer cells can manifest itself in two key ways: first, by major genetic events such as somatic copy number aberrations (SCNAs) and mutations; and second,

phenotypic variations in transcript and protein expression levels, and, not insignificantly, major metabolic rewiring. These processes are mediated by, for instance, epigenetic programming.

Microenvironment, Genetic and Metabolic Changes

The Microenvironment and Nutrients Influence Metabolic Changes in Tumors Besides genetic differences, individual tumors also show differences in phenotypes including metabolism (5, 8). Malignant solid tumors frequently encounter mild to severe hypoxia (oxygen deficiency) due to insufficient tumor microvasculature quality and quantity, culminating in impaired oxidative phosphorylation (OXPHOS). This cellular stress induces changes in tumor transcription, respiration and metabolism, promoting highly abnormal neovasculature formation and, ultimately, allowing increased cancer cell survival, proliferation, invasion and metastasis (9). These cancer cells, independent of their oxygenation, increase glycolysis and produce more lactate (the Warburg effect) (10). Instead, the Pasteur effect states that presence of oxygen would inhibit glycolysis (11), suggesting that under normoxic conditions cancer cells may prefer OXPHOS. Irrespective of these different effects, highly metastatic prostate cancer cell lines under normoxic conditions were shown to undergo glycolysis, while less metastatic lines were OXPHOS dependent (12). Similar effects have been seen in glioma cell lines (13). Although the apparent differences in energy metabolism in

different tumors are attributable to their intrinsic genetic, epigenetic, and microenvironmental characteristics, they may also represent distinct subtypes (9).

An example of hypoxia directly influencing metabolic programming via gene expression is the activity of pyruvate kinase isoforms M1 and M2 (PKM1/2), which are essential energy metabolism regulators critically involved in the final stages of glycolysis. Under hypoxic conditions, cancer cells expressing only PKM2 proliferate faster than those expressing only PKM1 (14). PKM2 exists as a less active dimer and a more active tetramer, the former being highly expressed in proliferating cancer cells and allowing upstream metabolites to accumulate to meet increased nucleotide, amino acid and serine biosynthesis needs (refer review (15)). This differential expression of PKM2 isoforms may represent distinct tumor subtypes.

Mutated Metabolic Genes as Cancer Drivers A more self-evident entwining of metabolism and genetics is when mutations in genes encoding metabolic enzymes are a first cancer "hit". Isocitrate dehydrogenase 1 (IDH1) is an enzyme that converts α-ketoglutarate (α-KG) to citrate in the tricarboxylic acid (TCA) cycle (16). However, mutated IDH1 can convert α-KG to 2-hydroxyglutarate (2HG), inhibiting enzymes controlling epigenetic methylation and consequently altering global gene expression (16). *IDH1* mutations are implicated in glioma (17) and acute myeloid leukaemia (AML) pathogenesis (18) and, interestingly, mutated *IDH1* enrichment partially defines the proneural glioblastoma subtype (6). Additionally, succinate

dehydrogenase (SDH) and fumarate hydratase, enzyme complexes involved in TCA cycle, also have links with cancer (original papers referred in (19)). Germline mutations resulting in the loss of one subunit of the SDH complex, SDHB, have been confirmed in a rare subset of renal cell carcinoma (20), and has shown association with therapeutic response to temozolomide in metastatic pheochromocytoma or paraganglioma (21).

Non-Metabolic Driver Mutations Affect Metabolism Clearly, cancer is not only caused by mutations in genes directly involved in metabolism, and first-hit mutations in non-metabolic genes also indirectly remodel tumor metabolism. Specific gene mutations often drive tumorigenesis in a large proportion of cases: *TP53* is the most frequently mutated gene in many cancers (22), and *KRAS* is a proto-oncogene mutated in over 90% of pancreatic ductal adenocarcinomas (PDAs) (23). In this latter case, advanced PDAs in a *Kras*-driven genetically engineered mouse (GEM) model are dependent on continued mutant *Kras* signaling, which stimulates glucose uptake and reprograms downstream anabolic metabolism (24). In addition, tumors with mutant *KRAS* are addicted to a non-canonical glutamine-supported metabolic pathway that drives their growth and upregulates aerobic glycolysis (25).

However, the requirement of mutant *KRAS* for tumor maintenance is heterogeneous, with well-differentiated epithelial cell lines – specifically the classical PDA subtype (see below) – being more reliant on KRAS signaling (3, 26). If mutations in particular genes like *KRAS* are

dominant cancer drivers in most cases of certain cancer types, what causes heterogeneity and different phenotypes in these tumors as the disease progresses?

Possible Origins of Tumor Heterogeneity Based on Driver Aberrations Cancer subtypes are mainly associated with distinct first-hit driver aberrations in normal cells (refer (27)), however, they can be genetic or metabolic aberrations (**Figure 1A – left and right panels**). Nevertheless, second-hit metabolic changes could lead to phenotypic heterogeneity in several ways in less heterogeneous tumors. For example, in cases in which a single mutated gene (e.g., *KRAS* in PDA) usually initiates tumorigenesis as a first hit, we hypothesize that tumor heterogeneity is instigated by a second hit to metabolic reprogramming induced by the microenvironment (**Figure 1A – left panel**). Inconsistent oxygenation and nutrient provision by imperfect microvasculature could lead to considerable microenvironment-based variability in different tumor regions (and between different tumors), prompting metabolic adaptation to local conditions that threaten cellular survival. For instance, metabolic reprogramming arising from obesity-related insulin resistance or excess reactive oxygen species (ROS) from mitochondrial metabolism could influence cancer cell proliferation and render them vulnerable to heterogeneity-causing mutations (refer (9)). Similarly, second-hit molecular or genetic changes could lead to distinct tumor subtypes in less heterogeneous tumors. Due to the wide-ranging

effects of metabolic and molecular reprogramming on phenotype, these adaptations could be the origin of different genetic and epigenetic subtypes.

Epigenetics Facilitates Metabolism-Transcription Feedback While both molecular and metabolic heterogeneity undoubtedly exist, their origin is debatable. Although tumor diversity is well represented by molecular/genetic profiles, this does not imply that the heterogeneity is completely molecular/genetic in origin. Gene expression is dependent on many factors including epigenetic modifications that regulate chromatin structure and DNA accessibility to transcriptional machinery. Epigenetic enzymes may be modulated not only by their own expression and that of their regulators, but also by the availability of metabolites they require as substrates or cofactors (28). For example, tet methylcytosine dioxygenase 2 (TET2) and lysine demethylase 3A (KDM3A) are two epigenetic enzymes that employ the metabolite α-KG as a cofactor. In the presence of mutant IDH1, which can convert α-KG to 2HG (16) (see above), 2HG competitively inhibits α-KG's binding to TET2 and KDM3A, influencing epigenetic marks (refer (29)). In this way, genetics, epigenetics and metabolism interact in a system to form a complex feedback mechanism.

Evidently, interactions between the tumor microenvironment, metabolism, and genetics are diverse and complex. The microenvironment regulates metabolic pathways via the epigenome and also influences them directly (refer (28)). Genomic aberrations (e.g. mutations) can affect

metabolic genes and non-metabolic genes with indirect actions on metabolism (17). The different outcomes of these interactions are a potential source of the heterogeneity that can lead to distinct cancer subtypes.

Context-Specific Molecular and Metabolic Heterogeneity

Transcriptomic PanNET Subtypes and Their Associated Metabolic Profiles Given the potential for metabolic heterogeneity to influence cancer cell phenotypes and, by extension, tumor subtypes, it is sensible to analyze transcriptomic and metabolic profiles together when attempting to stratify patients. By jointly analyzing mRNA and microRNA (miR) transcriptomes, we recently stratified human PanNETs into three molecular subtypes with distinct metabolic profiles (5). One subtype, the "insulinoma-like tumors" (IT; with increased insulin production), showed increased pyruvate carboxylase (*PC*) and cytoplasmic malic enzyme 1 (*ME1*) expression consistent with active pyruvate cycling, a process utilized by mature β cells to sustain glucose-stimulated insulin secretion. In contrast, the "metastatic-like primary" (MLP) subtype showed greater monocarboxylate transporter 1 (*SLC16A1/MCT1*) and hexokinase 1 (*HK1*) expression, which is suppressed in mature β cells (5). Transcriptomic PanNET subtypes appear to have distinct metabolic preferences.

Transcriptomic PDA Subtypes PDAs have often been regarded as homogeneous due to the overwhelming prevalence of driver *KRAS* mutations. However, for the first time, we

demonstrated that PDAs, like other cancers, can be classified into three gene expression subtypes using a 62-gene signature (PDAssigner) (3). One subtype, "classical PDAs", is characterized by high adhesion-associated, ribosomal and epithelial gene expression, and elevated *GATA6* expression (3), which is essential for pancreatic development (30).

The second PDAssigner subtype shows high expression of tumor cell-derived exocrine genes and was hence named "exocrine-like" (3) (corroborated by Moffitt *et al.* (31)). We took particular care to enrich cancer cells by microdissection for PDA subtyping to identify cancer-specific subtypes, and further validated the presence of exocrine-like subtype by performing immunohistochemistry to detect the cancer cell-specific expression of exocrine-like subtype proteins on gene expression subtype matched PDA samples (3). The presence of exocrine-like subtype was validated by Noll *et al.* (32), by deriving matched exocrine-like PDA patient-derived xenograft tumors and cell lines. In addition, they have shown this subtype to be resistant to tyrosine kinase inhibitors and paclitaxel via a novel mechanism, suggesting the requirement for different personalized approach for this cancer subtype (32).

The third subtype, the "quasi-mesenchymal PDAs" (QM-PDA), exhibits high mesenchymal gene expression, representing a possible association with cancer-associated fibroblasts/stroma. Moreover, we clearly demonstrated increased glycolytic gene expression, including *MCT1*, hexokinase 2 (*HK2*) and glucose transporter 3 (*SLC2A3*/*GLUT3*) in QM-PDAs. Hence, QM-

PDAs are a highly glycolytic PDA subtype with worse prognosis than the classical and exocrine-like subtypes (3). These subtypes were validated by independent studies involving patient PDA tumors (31-33) and cell lines (8), and by ourselves using GEM model-derived PDA cell lines (34). Although referred to as "basal-like" (based on a similarly-named breast cancer subtype) in Moffitt *et al.* (31), the QM-PDA subtype nomenclature was chosen to reflect the presence of both tumor and stromal genes in the signature (3, 31). Interestingly, the PDA subtypes in Bailey *et al.* (33) almost entirely conformed to our PDAssigner subtypes, except for an additional "immunogenic" subtype, where their a) "squamous" subtype represent our QM-PDA subtype, b) "pancreatic progenitor" represent our classical subtype and c) "aberrantly differentiated endocrine exocrine (ADEX)" represent our exocrine-like subtype (33). Conversely, there was lower concordance between the Moffitt and Bailey subtypes (33).

Importantly, classical and QM-PDA cell lines with different transcriptomes and metabolomes exhibit differential responses to two common therapies: classical PDA cell lines were more sensitive to erlotinib and QM-PDA lines to gemcitabine (3), despite increased mutant *KRAS* dependence in the classical subtype (3, 26). Patients with classical subtype tumors, therefore, may derive benefit from KRAS signaling related therapies, although this has yet to be realized clinically. However, this data provides clues as to why current clinical responses to erlotinib and gemcitabine in combination are heterogeneous in unselected PDA patients. Moffitt *et al.* have shown that basal-like (QM-PDA) subtype patients were associated with better response

to adjuvant therapy compared to those with classical subtype PDA (31), further suggesting personalized treatment options in this aggressive cancer type.

Metabolomic PDA Subtypes Complementary to the transcriptomic subtypes described above, metabolic profiling has also revealed three PDA subtypes (8): "glycolytic" PDAs (QM-PDAs), with elevated glycolysis and serine pathways, increased *MCT1* expression, and high glutamine incorporation into TCA cycle metabolites; "lipogenic" PDAs (classical PDAs), with lipid and electron transport chain metabolite enrichment and high lipogenesis gene expression, high oxygen consumption and mitochondrial content, and high glucose incorporation into TCA cycle metabolites; and "slow proliferating" PDAs low in amino acids and carbohydrates. These subtype-specific cell lines were also shown to have different responses to various metabolism-based inhibitors (8).

Putative Cell-of-Origin and Metabolic Phenotypes

An alternative hypothesis to distinct driver aberrations leading to different subtypes is that cancer cells with different cells-of-origin (27) or those which have undergone epithelial-

224 mesenchymal transition (EMT) and having distinct molecular and/or metabolic profiles
225 develop into distinct subtypes based on the cell's metabolic dependencies (**Figures 1B and C**).
226
227 ***The Cell-of-Origin/Phenotype of PanNETs*** Combined transcriptomic and metabolic profiling
228 can reveal patterns in phenotypes of cancer subtypes that are reminiscent of their normal
229 counterpart cells, probably reflecting different cellular origin. In PanNET, IT tumors are
230 clinically characterized as well differentiated, functional (secrete insulin) and low grade (have
231 low Ki67-based proliferation index), which infrequently metastasize, and share gene
232 expression and metabolism with mature islet β cells. Conversely, the proliferation rate in IT
233 cells is comparatively higher than in β cells irrespective of the infrequent somatic mutations in
234 tumors (5). Hence, ITs are likely to arise from more differentiated β cells. In this way, they are
235 probably similar to the exocrine PDA subtype (3) and enterocyte and goblet-like/metabolic
236 colorectal cancer (CRC) subtypes (see below) (4), which all retain characteristics of their
237 normal differentiated cells (all these are represented generally in **Figure 1B – left panel**).
238
239 In contrast, MLP subtype tumors are poorly differentiated and non-functional (i.e. no hormones
240 can be detected in the blood) and are associated with liver metastases and high tumor grades.
241 This subtype possesses a typical pancreatic stem/precursor cells or immature β cell

transcriptional signature and expresses genes associated with fibroblasts, stroma, stem cells, and hypoxia (see **Figure 1B**).

The Cell-of-Origin/Phenotype of Colorectal Tumors We discovered five clinically pertinent colorectal cancer (CRC) subtypes by mRNA profiling of 1,290 tumors ("stem-like", "transit amplifying" (TA), "enterocyte", "goblet-like", and "inflammatory" subtypes) (4). Comparisons with known colon-crypt cell type gene signatures revealed likely cell-of-origin/phenotype candidates (see **Figure 1B**). For example, the goblet-like and enterocyte subtype signatures were associated with those of the normal goblet and enterocyte cells (colon crypt top), while the stem-like subtype was associated with the crypt base, implicating these sites as the putative cell-of-origins for these subtypes. The stem-like subtype (with low differentiation marker expression) showed high stem cell, myoepithelial/mesenchymal and stromal gene expression.

Although these profiles were subsequently independently confirmed (35), other studies have concluded that CRCs can be divided into between three and six subtypes (36). Reconciliation of these subtypes has revealed that these classifications were in fact in broad agreement for four subtypes, with the remainder being further subdivisions of these "consensus molecular subtypes" (CMS) (36). One of these four CMS subtypes, which maps to our differentiated goblet-like subtype, was dubbed the "metabolic" (or CMS3) subtype (36) due to its enrichment

for several metabolic gene signatures, and was associated with high *KRAS* mutation frequency, whose influence on metabolism is discussed above (24, 25).

Epithelial and Mesenchymal Signatures in Cancer Subtypes EMT is a phenotypic switch in which cancer cells convert to a more invasive and metastasis-capable (mesenchymal) state. Since EMT is reversible it cannot simply be attributed to a genetic event, but instead is likely to represent a comprehensive reprogramming of the genetic, epigenetic, and metabolic profiles of the cell. This reprogramming is triggered by extracellular signaling, which results in genetic and metabolic adaptation to the microenvironment (refer (37)).

Tumor stratification into subtypes can also reveal an epithelial or mesenchymal classification (**Figure 1C**). In PDA, the classical subtype expresses high levels of epithelial genes including *CDH17* and *CEACAM6*, while QM-PDAs are enriched for the mesenchymal gene *TWIST1* (3, 8). In CRC, the stem-like (CMS4) subtype represents a mesenchymal phenotype, whereas goblet-like (CMS3) and enterocyte (subset of CMS2) CRCs represent epithelial phenotypes (4). Similarly, in PanNETs, the IT subtype exhibits differentiated cell-based markers, whereas MLPs have mesenchymal signature along with increased glycolytic genes. Moreover, the mouse MLP subtype can be further subdivided into those that express low or high insulin gene/protein ("MLP Ins-lo" and "Ins-hi"): the Ins-lo subtype probably originates from pancreatic stem/islet precursor cells (**Figure 1B**), while the Ins-hi tumors are likely to be the

result of epithelial-mesenchymal transition from mature β cells or the progression of β cell-derived IT tumors based on their gene expression profiles (see **Figure 1C**) (5). This suggests that the epithelial and mesenchymal phenotypes in PanNET subtypes are products of their cells-of-origin and a consequence of subsequent reprogramming between the epithelial and mesenchymal states (5). Nevertheless, it would be interesting to examine the interactions of genetic, epigenetic and metabolic factors that trigger EMT in these subtypes.

Concluding Remarks

Overall, there appears to be at least three broad divisions of the origins of tumor heterogeneity and subtypes based on metabolic, genetic and/or molecular changes. The first are subtypes with initial tumorigenic driver genetic or metabolic aberration(s) that give rise to different tumor subtypes (**Figure 1A – left and right panels)**. Nonetheless, an initial aberration as a first hit in their normal counterparts is probably followed by the gain of additional secondary genetic or metabolic aberration(s) that further drive progression and affect patient prognosis (**Figure 1A – left panel**). In the second main group of cancers, cell-of-origin determines the subtypes, with certain differentiated subtypes maintaining the transcriptomic and other important characteristics of their well-differentiated normal counterparts (38) (e.g. pyruvate cycling (5)) and being addicted to more normal cellular energy metabolism. Most of these subtypes have favorable prognosis (**Figure 1B – left panel**). Others, probably originating from stem/precursor cells, are likely to shift their energy metabolism toward glycolysis and other

malignant metabolisms, and have a poorer prognosis (**Figure 1B – right panel**). Finally, the malignant potential and metabolic reprograming are inversely correlated with differentiated cell-based marker expression, with epithelial and mesenchymal/stemness signatures resulting in different prognoses (**Figure 1C**). Whether this context-specific metabolic reprogramming in different subtypes is triggered from the outset of tumor proliferation or occurs as a result of cellular adaptation still needs to be determined.

Nevertheless, it has recently become clear that tumor heterogeneity influences therapeutic efficacy in a variety of cancer types. Stratifying patients into groups that best respond to treatment based on the individual tumor's driver molecular aberrations has had clinical success. For example, tamoxifen and trastuzumab are two drugs that have subtype-specific benefits in patients with estrogen-receptor and HER2-positive breast cancers, respectively (39, 40). Our recent work and that of others has indicated that these molecular indicators of drug/subtype specificity are also likely to exist in other cancers (3, 4, 6, 31, 35, 41, 42). However, whether metabolic changes, cell-of-origin and EMT could be exploited for personalized/precise cancer therapies requires increased attention.

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Figures

Figure 1. Various origins of tumor heterogeneity. (A) Driver aberrations determine the characteristics of the ensuing tumor and, consequently, two driver aberrations (genetic or metabolic; aberration-1, e.g. *KRAS* mutation, shown as a red star inside the cell in the left panel, and a different one - aberration-2, e.g. an *EGFR* mutation, shown as a yellow star inside the cell in the right panel) can result in distinct tumor subtypes (Subtypes A or B). Subsequently, a second metabolic or genetic hit can determine tumor subtypes (left panel). A homogeneous tumor derived from a cell with a particular tumor-initiating driver aberration-1 can acquire a further metabolic or genetic hit for subsequent tumor progression. The nature of the second hit determines the associated characteristics of the progressing tumor subtype, leading to heterogeneity (Subtypes C or D). **(B)** Heterogeneity can arise depending on the cell-of-origin. In certain cases, tumors that arise from well-differentiated cells (e.g., β cells) can result in a subtype (Subtype E) that maintains both the metabolic and genetic profiles of the original cell-of-origin, and mostly have favorable prognosis (shown with a Kaplan-Meier curve). On the other hand, those tumors arising from stem/precursor cells with fewer markers of differentiation (Subtype F) probably have fewer metabolic and genetic characteristics of their parental cells and have a poorer prognosis (shown with a Kaplan-Meier curve). **(C)** EMT can lead to distinct tumor subtypes. Tumors originating from epithelial or mesenchymal cells can result in distinct subtypes (Subtypes G and H, respectively) with different prognoses (shown with a Kaplan-Meier curve). EMT is shown with a reversible arrow.