Tumor Heterogeneity in Pancreatic Adenocarcinoma

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Abstract

Pancreatic adenocarcinoma is one of the deadliest malignancies worldwide, mainly due to frequent diagnosis at an advanced stage and its strong chemoresistance. Tumor heterogeneity is evident at the histological level, both between tumors and even within a tumor. Recent high-throughput analyses have confirmed that intertumor heterogeneity is greater than intratumor heterogeneity that is mostly driven by successive catastrophic genetic events in the early stage and by epigenetic modifications in the metastatic stage. While this heterogeneity may complicate the search for a universal cure, these analyses have distinguished several subtypes at the genomic, transcriptomic, and metabolomic levels that offer, for some, new therapeutic opportunities.

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Pancreatic cancer heterogeneity starts with its denomination. Indeed, “pancreatic cancer” encompasses several histological types, with the most common being pancre-

atic ductal adenocarcinoma (PDAC), which will be discussed below. One should keep in mind that other epithelial tumors, such as acinar cell carcinoma or neuroendocrine tumors, also exist. They have completely different molecular profiles and clinical outcomes and should therefore not be likened to PDAC. PDAC has a very poor prognosis, with a 5-year survival rate of around 7%. This is due on the one hand to most patients presenting at an advanced stage at the time of diagnosis when “curative” treatment (i.e., surgery) is no longer indicated and on the other hand to the lack of efficacy of the current chemotherapies. Thus, a better molecular characterization of PDAC is urgently needed in order to identify the key drivers and pathways involved in pancreatic carcinogenesis and potential therapeutic targets. Recently, several “omic” analyses (i.e., genomic, transcriptomic, and metabolomics) have highlighted the high degree of heterogeneity of PDAC between individuals (intertumor heterogeneity) but also within the same tumor (intratumor heterogeneity). In this review, we will first discuss intertumor heterogeneity at the histological and molecular levels looking at both tumor and stroma compartments. We will then review the knowledge on PDAC clonal evolution to understand intratumor heterogeneity within the primary tumor and between the different metastatic localizations.

Intertumor Heterogeneity

Every pathologist knows that PDAC is a very peculiar tumor with an abundant stroma and a highly heterogeneous histological aspect. Recent high-throughput analyses have unraveled the diversity of PDAC at multiples molecular levels (DNA, RNA, etc.), but the correlations between histological aspects and molecular subtypes are still lacking.

Histological PDAC Subtypes

PDAC histological features are highly heterogeneous. The WHO classification describes several PDAC subtypes. Ductal adenocarcinoma is the most common form (85%), followed by adenosquamous carcinoma (0.4–10%), colloid carcinoma (2–5%), and medullary, hepatoid, signet ring, undifferentiated anaplastic, and undifferentiated with osteoclast-like giant cell carcinomas (all <1%). In addition, PDAC often displays different patterns (clear cell, foamy cell, large duct, intestinal, micropapillary, and cystic papillary) that may coexist within the same tumor [1–8] (Fig. 1). The true impact of these subtypes on the prognosis is still unclear, with important discrepancies between unicenter reports and registry studies. For instance, adenosquamous carcinomas have been reported to have a poorer prognosis compared to ductal PDAC, but SEER data demonstrate that there is in fact no difference [9–11]. Similarly, colloid carcinomas, often derived from intraductal papillary and mucinous neoplasms, have been reported to have a better prognosis [12, 13]. As these subtypes are rare, their molecular features have not been completely elucidated and they rarely impact clinical care. Adenosquamous carcinomas have been reported to harbor a mutational pattern fairly similar to that of ductal PDAC, yet they most often correspond to the aggressive basal-like subtype (see below) [14]. Whether the histological subtype should be taken into account on a diagnostic biopsy to tailor treatment remains to be demonstrated. The medullary subtype or tumor with medullary-like features (poorly differentiated tumors with a syncytial growth pattern and an important lymphocyte infiltration) should be recognized as they may present a deficiency in the DNA mismatch repair system and therefore be sensitive to immunotherapy [15, 16].

Genomic PDAC Subtypes

The 4 most frequently altered genes in PDAC, i.e., *KRAS* (90%), *TP53* (60–70%), *CDKN2A* (40–50%), and *SMAD4* (30–40%), have long been known, and whole exome or genome analysis did not reveal new key driver

genes with a frequency above 20%, confirming the important mutational landscape heterogeneity of PDAC [17–21]. None of these frequently mutated genes individually defines a clear subtype, although some, like *CDKN2A*, *SMAD4*, or *MYC*, have been shown to have a prognostic impact [22–24]. It appears that some mutations may be more frequent within one histological variant, such as *MYC* amplification in adenosquamous carcinomas, but this remains to be demonstrated in large series [25]. Otherwise, these high-throughput analyses have defined 4 subtypes at the genome level: (i) a stable subtype (20%) with less than 50 structural variations, (ii) a locally rearranged subtype (30%) with significant focal events located on 1 or 2 chromosomes, (iii) a scattered subtype (36%) with widely distributed nonrandom chromosomal damage (<200), and (iv) an unstable subtype with extended (>200) structural variations [19]. Based on published mutational signatures, Connor et al. [21] identified 4 main PDAC subtypes that partially overlap with those described above: (i) an age-related subtype (70%), (ii) a double stand break repair group (11%) that partially overlaps with the unstable subtype, (iii) a mismatch repair group (2%), and (iv) a group of unknown etiology. While these subtypes cannot be easily defined in routine practice, they may have an important theranostic value. For example, the unstable subtype/double stand break repair group seems to be particularly sensitive to platinum-based therapy, it may be sensitive to PARP inhibitor, and it appears to have an increased T CD8 infiltration which may render these tumors sensitive to immunotherapies [19, 21]. Of note, a third of the patients within this group had no anomaly identified in the DNA repair genes, making genetic screening based only on mutational analysis insufficient. The remaining two thirds had germline or somatic mutations with genes known to be involved in familial forms of PDAC, such as *BRCA1/2*, *PALB2*, etc. Unfortunately, the histological analysis of these familial PDAC showed no particularity [26].

Identification of PDAC with a DNA double stand break repair deficiency is very challenging in routine practice. A targeted DNA sequencing approach would be the most appropriate technique and would work in formalin-fixed paraffin-embedded samples, even in fine-needle aspiration biopsies, but it would miss a significant number of patients. Whole genome sequencing is the gold standard but it is still very expensive and requires dedicated bioinformatics structures for its analysis. However, it has been reported that such an approach in breast cancer could work on formalin-fixed paraffin-embedded biopsies with a high sensitivity [27]. Additionally, impor-

Fig. 1. Morphologic heterogeneity of pancreatic adenocarcinomas. **a** Well-differentiated “classical” pancreatic ductal adenocarcinoma (PDAC), characterized by a proliferation of well-defined small glands, lined by tumor cells with basal nuclei. Note the abundant fibrous (desmoplastic) stroma. HE. $\times 20$. **b** Adenosquamous carcinoma characterized by a proliferation with both ductal and squamous differentiation. HE. $\times 20$. **c** Colloid carcinoma (mucinous noncytic carcinoma) composed of large extracellular stromal mu-

cin pools partially lined by well-differentiated cuboidal to columnar neoplastic cells. HE. $\times 10$. **d** Undifferentiated carcinoma with osteoclast-like giant cells, composed of round to spindle shaped, highly pleomorphic neoplastic mononuclear cells and large, non-neoplastic multinucleated histiocytic giant cells. HE. $\times 20$. **e** Intestinal variant of PDAC composed of glands lined by cuboidal to columnar neoplastic cells of intestinal differentiation. HE. $\times 20$. **f** PDAC with a micropapillary architecture. HE. $\times 20$.

tant DNA methylation profile modifications have been reported in PDAC compared to normal tissue, but no subtypes have been individualized yet [28].

Transcriptomic PDAC Subtypes

The heterogeneity seen at the DNA level is also present at the RNA expression level, although there is no overlap between the two. Cancer subtyping using the RNA signa-

ture is already available in routine practice for breast cancer and it has been proposed in other cancer types, and this may represent an important opportunity to tailor patient treatment. So far 3 groups have proposed RNA-based signatures of PDAC. Collisson et al. [29] first proposed a 3-group classification: (i) a quasi-mesenchymal subtype (30%), the most aggressive, (ii) an exocrine-like subtype (35%), with an intermediate prognosis, and (iii) the clas-

sical subtype (35%), with the best prognosis [29]. Interestingly, this classification has been validated in an independent data set [30]. Bailey et al. [18] proposed a fairly overlapping 4-group classification: (i) a squamous subtype (quasi-mesenchymal), (ii) aberrantly differentiated endocrine exocrine (ADEX; exocrine-like), (iii) pancreatic progenitor (18%) (classical), and (iv) immunogenic (17%) (classical). Squamous/quasi-mesenchymal PDAC had an increased expression of EMT genes, hypoxia markers, and metabolic reprogramming; ADEX/exocrine-like PDAC expressed exocrine secretion and beta cell development genes; pancreatic progenitor PDAC expressed early pancreatic development gene sets, and immunogenic PDAC were infiltrated by B and T cells. The last proposed classification from Moffitt et al. [31] is simpler, separating PDAC into: (i) a more aggressive basal-like subtype and (ii) a classical subtype. The overlap with the other classifications was poor. All squamous/quasi-mesenchymal PDAC were classified as basal-like but the remaining 3 subtypes were classified as either basal-like or classical. To date, no correlation has been established between these subtypes and any histological feature, except for the squamous signature that is more frequent in adenosquamous carcinomas. Noll et al. [32] proposed that the classification of Collisson et al. [29] could be recapitulated by immunohistochemistry by assessing the expression of HNF1A and KRT81. This requires external validation and no subtype is currently assigned to double-positive tumors, but it may represent an interesting tool to subtype tumors and could have a major clinical impact. For instance, identification of the aggressive subtype may help to select patients who would benefit the most from neoadjuvant therapy prior to surgery, and the immunogenic subtype may be a candidate for immunotherapy. In addition to gene expression signatures, many miRNA have been described to have a prognostic value but few studies have tackled miRNA expression in a nonsupervised manner. Namkung et al. [33] described 3 subtypes based on miRNA profiles. One, representing 20% of the patients, was associated with a poor prognosis and p53/COX2 pathway modulation [33]. No integrated analysis has yet been performed to address whether these subtypes overlap with the mRNA classifications described above. A global agreement on the subtypes and the defining genes needs to be reached in order to implement the use of these signatures in routine practice. Although it is more complicated to work with RNA than with DNA, especially in formalin-fixed paraffin-embedded samples, it is now routinely done in breast cancer management, for example.

Metabolomic PDAC Subtypes

Metabolomic heterogeneity is also a feature of PDAC. By analyzing the metabolomic profile of 38 cell lines, Daelmen et al. [34] described 3 types of tumors: (i) a slow proliferative subtype (34%) with a low level of amino acids and carbohydrates and a long doubling time, (ii) a glycolytic subtype (27%) with a high level of intermediates from the glycolysis and serine pathways and reduced oxidative phosphorylation, and (iii) a lipogenic subtype (39%) enriched for various lipid and oxidative phosphorylation metabolites [34]. Interestingly, these subtypes overlap with the RNA classification. All glycolytic cell lines harbor a quasi-mesenchymal phenotype and most lipogenic cell lines a classical phenotype. In addition, these subtypes display a differential sensitivity to drugs targeting glycolysis or lipogenesis. These metabolic subtypes need to be confirmed in primary human tumors, but metabolism targeting is a very active field, with many new drugs being tested.

PDAC Stroma Subtypes

In addition to tumor cell heterogeneity, it is now becoming clear that there is more than one type of stroma. While its pro- or antitumor role remains controversial, identification of the different types of components and their relative distribution may help to reconcile the discordant results obtained so far [35–37]. One obvious varying parameter for pathologists is the abundance of stroma. Erkan et al. [38] proposed an activated stroma index that relies on the area occupied by α SMA+ stromal cells/the area occupied by collagen. While considerable variations could be seen between tumors, those with a low collagen abundance and a heavy α SMA+ stromal cell infiltration had a worse prognosis, a finding confirmed by others [39]. In addition to the raw number of activated fibroblasts, their functional heterogeneity and distribution may dramatically impact the tumor outcome. This has been clearly suggested in other cancer types such as breast cancer, where at least 4 fibroblast subtypes have been described [40]. In PDAC, a definitive characterization of fibroblast subtypes is still awaited, but some studies have already suggested that there are at least 2 types of cancer-associated fibroblasts (CAF). Öhlund et al. [41] recently described one type of CAF α SMA+ located close to the tumor cells that was responsible for the desmoplastic stroma production, and one type of inflammatory CAF located further away from tumor cells that secreted interleukins (IL) such as IL-6, IL-1, and IL-11 and chemokines. Similarly, it has been shown that differential expression of transcription factors such as SNAIL/ZEB1/

ZEB2 in stromal cells could either trigger local budding of tumor cells or favor migration and distant dissemination [42]. In addition to CAF, there is probably a certain heterogeneity within pericytes and inflammatory cells populations. Global transcriptomic profiling of PDAC stroma has suggested that there are 2 types of stroma, i.e., a “normal” type with classical stellate cell genes and an “activated” type with macrophages, metalloproteinase genes, and transcription factors [31]. Patients with activated stroma had a shorter survival and this information could be combined with the tumor subtype to further refine their prognostic value. Evaluation of the stroma is not possible on fine-needle aspiration and it remains a challenge on biopsy. New needles with a cutting edge should improve the size of the biopsy fragments. This will become a priority, as new trials are now targeting the stroma and require its characterization to stratify patients. For instance, an ongoing phase 3 trial using pegylated hyaluronidase requires a new biopsy with enough material to assess the level of hyaluronic acid in the stroma [43].

Intratumor Heterogeneity

In addition to intertumor heterogeneity, intratumor heterogeneity plays a key role in tumor progression and drug resistance. This heterogeneity can be spatial, within the primary tumor, between the later and the metastases, and even between metastases themselves. Temporal heterogeneity adds another level of complexity and becomes crucial when clonal selection is induced by therapies. This raises an important issue about the sample (in terms of localization and time) that should be used to determine the subtypes described above. Tumor evolution is therefore the key to understanding intratumor heterogeneity. High-throughput genetic analyses combined with multiple sampling have assisted in tackling these questions.

Primary Tumor Heterogeneity

Mirroring the histological intratumor heterogeneity, early studies in xenografted animals have demonstrated that the transcriptomic profile differs (by >1,000 genes) between the center and the periphery of the tumor. Gene signatures related to the cell cytoskeleton and motility were upregulated at the periphery while regulation of transcription, response to stress, and carbohydrate metabolism signatures were upregulated at the center [44]. Harada et al. [45] microdissected 3–4 adjacent tumor glands from 20 primary PDAC and compared their genomic profile by comparative genomic hybridization. While most abnor-

malities were shared by all of the glands, some were specific, clearly demonstrating the extent of tumor heterogeneity [45]. Whole genome or exome sequencing of primary and metastatic lesions may suggest at first sight an important heterogeneity at the metastatic level, but some studies have shown, by sequencing of multiples area of the primary tumor, that most subclones were already present in the pancreatic localization [46]. Such analyses have also revealed a poor correlation between the successive subclonal evolution and their geographic localization within the primary tumor. Probably because of a differential expansion rate, subclones a few centimeters away from each other may be closer genetically than directly adjacent subclones. This could be viewed as a major obstacle when searching for alterations predictive of drug efficacy, yet it has been shown that all of the major tumor driver alterations are present in all subclones [47]. The biological significance of the additional alterations specific to subclones, that represent about 35% of the mutational burden, has yet to be proven to play a role in tumor progression.

Tumor Evolution and Heterogeneity

The classical view of cancer evolution is through an accumulation of genetic alterations from low-grade preneoplastic lesions to carcinoma through high-grade lesions. Studies in animal models have shown that early lesions such as acinar-to-ductal metaplasia were polyclonal in 25% of cases in contrast to intraepithelial neoplastic lesions (PanIN) that were mostly monoclonal [48]. Comparison of primary tumors and metastases revealed that most genetic events, mutations, and larger chromosomal rearrangements occur within the primary tumor and rather early in the evolution [46, 47, 49]. Notta et al. [49] recently challenged this long multistep process. Their genetic analyses suggested that two thirds of the tumors went through catastrophic chromosomic events (4–5) during mitosis, such as chromotrypsis (multiple clustered chromosomal rearrangements occurring in a single event involving one or a few chromosomes) and later polyploidization (half of the tumors) (Fig. 2). They confirmed that most driver mutations occurred early and that multiple oncogene or tumor suppressor genes were amplified or deleted, respectively, at once during these events. This would explain the relatively low heterogeneity for driver events but suggests that progression from PanIN-1 to cancer without passing by all of the increasing dysplastic stages could be very rapid. In addition, the presence in all tumor cells of these large chromatin remodelings suggests that they confer an important proliferative and survival advantage to rapidly dominate the tumor.

Fig. 2. Phylogenetic tree illustrating the tumor evolution and intratumor heterogeneity in pancreatic adenocarcinomas (adapted from Makohon-Moore et al. [47]). Evolution within the primary tumor is punctuated by genetic catastrophic events responsible for multiple gene alterations together with point mutations responsible for most driver alterations. Further evolution, especially within the metastases, seems to be mostly regulated and driven by epigenetic remodeling. Time is represented on the vertical axis, and divergence is represented on the horizontal axis. Dotted arrows represent additional mutations. SC, subclone; Liv M, liver metastasis; Perit M, peritoneal metastasis; Lung M, lung metastasis.

Metastasis Heterogeneity

Once again, genetic studies have offered key insights into metastases, which represent a large part of the tumor burden and are present at the time of diagnosis in more than half of the patients. These studies have suggested that most liver and lung metastases were clonal and that the founding subclone was present within the primary tumor, with little to no trans-metastases seeding [48]. As mentioned above, all driver events are common with the primary tumor, but “global metastasis-promoting genes” have yet to be identified [46, 50, 51]. It rather appears that each metastasis may be “unique” in its mechanism which may impact the response to therapy. On the bright side, it can be assumed that theranostic mutations, if they in-

volve a key driver gene, could be assessed at any localization. In addition, it appears that the described genomic and transcriptomic subtypes are conserved in the metastases, but the studies were performed on a very small number of cases and these results need to be confirmed in larger series [18, 31].

An interesting emerging point is that peritoneal metastases (local dissemination) seem to be different from liver and lung metastases (hematogenous dissemination). While their genomic landscape is very similar, it has appeared that they had very different epigenomic reprogramming [52]. Depending on the localization, the distribution of the different histone types and modifications varies and this results in a wide modification of

genome methylation and gene expression. As epigenetic reprogramming and metabolism appear to be strongly linked, it was surprising to find that distant metastases had a metabolism overtly turned towards glucose compared to peritoneal localization. This may be of clinical relevance when selecting patient for trials with new metabolic-targeting therapies. In a large proteomic analysis performed in a patient with multiple metastatic lesions, the authors confirmed that 25% of the proteomes were variable between metastases, with an enrichment in receptor activity, signal transduction, and extracellular matrix proteins [53]. Again, lung and liver metastases appeared closer to each other than to peritoneal metas-

tases. Even greater differences were also observed in the phospho-proteome, with possible important therapeutic implications.

In conclusion, it is now clear that PDAC is a highly heterogeneous tumor. Intertumor heterogeneity is greater than intratumor heterogeneity that is mostly driven by epigenetic modifications. While this may complicate the search for a universal cure, recent high-throughput analyses have distinguished several subtypes that offer some therapeutic opportunities. Defining these subtypes will be a major challenge for the pathologist, but so far studies correlating histological features with molecular signatures are still lacking.

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