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Genomic, Proteomic, and Metabolomic Analysis of Normal Adjacent Tissue in Cancer: A Review

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ABSTRACT

Cancer is the second leading cause of death in the United States. In the early stage of the disease surgical removal is the primary therapy. The cardinal principle of cancer surgery is complete removal of the tumor with an area of healthy tissue around it. In recent years extensive research has brought to light the critical role played by host stromal cells in the development, progression, and spread of cancer. However non-stromal host cells adjacent to the tumor as seen by microscopic morphology were considered normal. In recent years, the so called normal adjacent tissue (NAT) when studied at genomic and proteomic level is far from normal. These changes are detectable in studies involving pH, allelic imbalance, telomere length, stromal behavior, transcriptome, and epigenetic alterations. These significant genotypic and phenotypic changes are detectable up to one centimeter from the margin of the tumor, thus the histological normalcy does not equal biological normalcy. This article reviews the data on the abnormalities noted at molecular and metabolic levels and their potential role in the malignant process.

KEYWORDS

Transcriptome; Telomere length; Stromal behavior

INTRODUCTION

The American Cancer Society estimates that in 2020 nearly 1.9 million new cancer cases will be diagnosed and a little of 600,000 people will die from the disease [1]. Surgical resection is the first line of therapy for most malignant tumors which present in early stage, localized and in an area amenable for surgical resection. The cardinal principle of surgical cure is total removal of neoplastic tissue with an area of healthy tissue around the tumor. Frozen section is used to confirm clear margins in situations where there is uncertainty, while the patient is still on the operating table. The risk of local recurrence is increased with close or positive surgical margins. Local recurrence can significantly impact the quality and quantity of patient's life. The extent of the tumor has traditionally been defined by the microscopic appearance as determined by the pathologist. The tissue beyond the edge of the tumor is labeled as normal.

Solid tumors besides containing malignant cells also have many different non-malignant cells in their micro-

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environment. These include immune and endothelial cells combined with a heterogeneous population of stromal cells including cancer-associated fibroblasts. The interactions between tumor and stromal cells substantially affect tumor cell biology. These cells have the potential to either promote or inhibit development and progression of malignant tumors [2].

Normal non-stromal adjacent tissue has attracted less attention than the role stroma in the evolution and progression of malignancy. However, recently attention has been directed to the so called normal adjacent non-stromal cells. They have been designated as "normal adjacent tissue (NAT)".

MATERIALS AND METHODS

A literature search was performed using the following keywords: normal or healthy or cell and near or next or peritumoral and cancer or tumor or malignant.

The search was performed on PubMed (PubMed includes Medline and Medline Plus), Cochrane Library, and Trip database from the years 2014-2020 in English language. Selected references from these publications were also reviewed.

Characteristics of normal adjacent tissue: How normal is the "normal" tissue adjacent to cancer cells?

Cancers progress when cellular and extracellular factors promote cell growth, proliferation, invasion/migration, angiogenesis, and glucose metabolism, while cancer progression is inhibited when factors suppress these activities and promote apoptosis. Importantly, there is increasing evidence of extensive crosstalk between tumors and the "normal" tissue surrounding these tumors termed normal adjacent to tumor (NAT)-to the extent that scientists are now questioning whether NAT tissue is truly normal and to what extent NAT may play a role in the patient's prognosis.

Since the 1990s, the region immediately surrounding tumors (up to 1 cm from the tumor margins) have been shown to exhibit several characteristics distinct from those of cancer-free tissue, including altered pH [3], allelic imbalance [4], different stromal behavior [5], and transcriptomic and epigenetic aberrations [6]. However, few studies have focused on NAT tissue at the molecular level to determine whether this assumption is, indeed, correct. This is partly attributable to the shortage of age- and site-matched samples from healthy individuals. As a result, the majority of the limited numbers of studies that have characterized NAT tissue relative to healthy tissue have focused on breast cancer, as healthy breast tissue samples are available from cancer-free individual's undergoing breast reduction surgery [7-9]. The findings of these and more recent studies are indicating that, in fact, the normal tissue adjacent to tumors may not be normal, even if it appears normal under the microscope. Rather, NAT has characteristics that distinguish it from both healthy and cancerous tissue, putting it in "a unique intermediate state" [10].

Transcription analysis of NAT reveals that it is far from normal even though the morphology under the microscope appears to be normal. These changes are multiple including pH, allelic imbalance, telomere length, stromal behavior, transcriptome, and epigenetic alterations [2,3,8,10,11]. These significant genotypic and phenotypic changes are detectable up to one centimeter from the margin of the tumor, thus the histological normalcy does not equal biological normalcy [10]. Analysis NAT may potentially provide prognostic information and also open new avenues for therapeutic intervention.

Theories regarding the intermediate status of NAT

There are currently three main hypotheses regarding how the NAT tissue acquires an intermediate status between normal and malignant tissue. These are: 1) the tumor cell contamination theory, proposed to explain the high

recurrence rates of breast cancer after surgery and suggesting that tumor cells enter NAT tissue and "contaminate" it [12]. 2) the field cancerization theory, proposed to explain the multifocality of primary tumors and suggesting that multiple genetic aberrations appear independently of each other in tissue exposed to factors promoting tumorigenesis (e.g., carcinogens), leaving NAT tissue in a pre-neoplastic state composed of histologically normal but genetically altered cells [13,14]; and 3) the tumor microenvironment (TME) theory, proposed to account for the aberrant signals observed in NAT compared to cells from cancer-free individuals and suggesting that normal tissues are subjected to and influenced by signals from the TME [15,17]. These three theories all attempt to explain the presence of indicators of precancerous changes in histologically normal tissues.

A 2016 study on breast cancer transcription profiles from The Cancer Genome Atlas (TCGA) suggests that the truth may lie between the field cancerization theory and the TME theory [18], through the so-called "etiologic field effect" proposed the year earlier [19]. This theory is an extension of the traditional field cancerization theory combined with the TME theory. This concept offers a coherent model of NAT status, in which the TME propagates aberrant signals from tumor cells at the genomic, epigenomic, transcriptomic, proteomic, and/or metabolomic level. The findings of this study suggest that not only does NAT tissue provide distinct information from that of tumor samples, but it may also provide information of prognostic value.

Interaction between Cancer and NAT

There are multiple types of interactions between the malignant tissue, the tumor microenvironment, and the "normal adjacent tissue". These are discussed as follows:

Crosstalk between NAT tissue and cancer cells through the tumor microenvironment (TME)

The TME is a complex and dynamic system. Its cellular composition is heterogeneous and includes not only cancer cells but also other cells such as adjacent normal cells, fibroblasts, infiltrating immune cells, and angiogenic vascular cells [20]. In addition, the TME includes various secretions from these cells such as growth factors, cytokines, and extracellular matrix. In this way, tumor cells interact with and dynamically remodel their microenvironment to make it more conducive to tumor proliferation and invasion into the NAT tissue, as well as promoting metastasis to more distant sites [2].

There is marked spatial and temporal variation in blood flow due to disordered vascular development, which, in turn, creates complex gradients of key substrates and metabolites (oxygen, glucose, and H^+), as well as growth and regulatory factors, which are primarily transported to and from the tumor tissue by the vascular system [21]. As a result, the TME exhibits typical characteristics: the pH is acidic, oxygen levels are variable with a tendency towards hypoxia, substrates are in short supply, and there are abundant toxic reactive oxygen and nitrogen radicals [3].

However, these effects are exerted both ways, as cancer cells also play an active role in shaping their environment- a strategy termed "niche engineering [11]". For example, tumor cells often release increased levels of growth factors, which diffuse through the stroma and cause characteristic changes in vascular growth. Cancer cells also commonly alter their environment through the preferred use of anaerobic glucose metabolism (i.e., glucose metabolism to lactic acid) even in the presence of normal oxygen concentrations. This is termed the "Warburg effect [22]".

Various factors mediate TME interactions, including soluble factors, cytokines, microRNAs, and extracellular vesicles (including exosomes) derived from tumor or

stromal cells [2]. Microorganisms, such as bacteria and viruses, may also be involved in microenvironment remodeling.

TME effect on NAT tissue through the promotion of epithelial-to-mesenchymal transition

Epithelial-to-mesenchymal transition (EMT) is a normal biological process through which epithelial cells convert into a mesenchymal phenotype, but it is also a key process in tumorigenesis, metastasis, and drug resistance [23]. In the metastasis of carcinoma, for example, epithelial cells at the invasive front of a carcinoma acquire an enhanced migratory phenotype by means of EMT [24].

During EMT, cadherin-mediated cell-cell adhesion is disrupted and a dramatic re-organization of the cytoskeleton occurs, allowing the affected cells to acquire migratory and invasive capabilities [23]. EMT is also reversible via the mesenchymal-to-epithelial transition (MET), which is thought to affect circulating cancer cells when they reach a desirable metastatic niche and begin to develop secondary tumors.

Recent research has indicated that the TME promotes EMT, both in tumors and NAT tissue. For example, a 2015 study used 3D co-cultures of normal epithelial cells and carcinoma cells, and the researchers found that the cancer cells produced a protease that cleaved E-cadherin from the surface of the normal cells [25]. This soluble E-cadherin associated with epidermal growth factor receptor, promoting the EMT of normal epithelial cells. Targeting EMT in tumors and NAT tissue is, therefore, an attractive target for anticancer therapy [23].

In cancers, EMT inducers are hypoxia, cytokines and growth factors secreted into the tumor microenvironment, stroma crosstalk, metabolic changes, innate and adaptive immune responses, and treatment with antitumor drugs [23]. The switch in gene expression from the epithelial to mesenchymal phenotype is triggered by complex regulatory networks involving transcriptional control by transcriptional factors, non-coding RNAs (miRNAs and long non-coding RNAs), chromatin remodeling and epigenetic modifications, alternative splicing, post-translational regulation, protein stability, and subcellular localization. Many of these factors exert their effects via the TME.

TME effect on NAT tissue through cell fusion

Cell fusion is a process whereby two or more cells become one by membrane fusion [20]. Cell fusion is essential for many normal biological processes, but it is also implicated in tumor initiation and progression. Researchers have shown that cells in NAT tissue can, through cell fusion, acquire the tumorigenic mutations, epigenetic aberrations, and other malignant characteristics of tumor cells, thereby facilitating cancer progression. The process of cell fusion in tumors and NAT is promoted by factors within the TME.

Cell fusion contributes to tumorigenesis through the production of polyploid cells, where the extra chromosomes lead to genetic instability and result in malignant transformation [20]. In terms of tumor progression, cell fusion is involved in cancer stem cell formation, high invasiveness acquisition, TME remodeling, epithelial- mesenchymal transition (EMT), drug resistance, and tumor angiogenesis, which are closely related to the growth, invasion, and metastasis of tumors.

Several TME factors promote aberrant cell-cell fusion [20]. For instance, the TME is typically deficient in oxygen and nutrients and is characterized by a relatively low pH and a chronic inflammatory state. The hypoxia and chronic inflammation signaling pathways associated with the TME, such as the matrix metalloproteinase (MMP) and tumor necrosis factor (TNF) pathways; promote the fusion of tumor cells with other tumor cells, cells in the NAT, and stromal cells.

Thus, cell fusion offers an efficient avenue for cancer progression into the NAT at a higher rate than that offered by cancer cell proliferation alone [20].

Genomic changes in NAT

DNA is continuously damaged by genotoxic agents generated either in the environment (e.g., UV light, ionizing radiation, and chemical exposure) or intracellular (e.g., reactive oxygen species as byproducts of routine metabolic processes) [26]. The resulting genetic aberrations include genomic rearrangements, copy number variation, somatic mutations (SNPs and indels), chromosomal abnormalities such as aneuploidy or tetraploidy, and uracil misincorporation into DNA, and these can all lead to tumorigenesis. This is particularly the case for mutations in the cell cycle checkpoint and DNA repair genes [27].

As is emerging with other hallmarks of cancer, NAT appears to exhibit an intermediate status in terms of harboring genetic aberrations linked with cancer. A 2017 study of hepatocellular carcinoma (HCC) using tumor tissue, NAT, and normal blood samples from three patients with HCC identified several mutations that were present at intermediate frequency in NAT relative to the tumor and normal blood samples [28].

Genomic aberrations in NAT tissue have potential as prognostic markers. For example, *SRARP* and *HSPB7* are tumor suppressor genes located 5.2 kb apart on chromosome 1p36, and these genes are commonly inactivated in cancers through chromosomal deletions or epigenetic silencing [29].One study found that DNA hypermethylation and lower expression of *SRARP* in NAT tissue was a predictor of poor survival, suggesting that SRARP inactivation is an early event in carcinogenesis [29].

Differences at the transcriptomic and proteomic levels

There have been several ambitious, multi-pronged studies looking at the differences in expression at mRNA and protein level between cancerous tissues and NAT, complemented by bioinformatics approaches to analyze the likely pathways implicated by these differentially expressed genes (DEGs) [30-33]. These bioinformatics approaches include the use of databases such as KEGG (Kyoto Encyclopedia of Genes and Genomes) and GO (Gene Ontology), as well as tools to map likely protein-protein interaction networks. It is hoped that such studies will provide an improved understanding of carcinogenesis and provide new therapeutic targets.

However, few studies have compared NAT, cancer tissue, and healthy tissue from cancer-free controls with respect to mRNA and protein expression. A 2005 study ahead of its time questioned the use of NAT as the baseline against which comparison of tumor tissue is made [34]. They compared the mRNA expression profiles of primary prostate cancer (tumor), NAT, and normal tissue from cancer-free donors. They identified unique gene expression profiles for each of these sample types. The tumor vs. cancer-free donor expression profile exhibited more DEGs than the tumor vs. NAT profile. When donor tissue was used as the baseline, similar DEG genes were found in both tumor and NAT tissue. Significantly, both tumor and NAT exhibited significant up-regulation of proliferation-related genes including transcription factors, signal transducers, and growth regulators compared to donor tissue. Importantly, these genes were not picked up in a direct comparison of tumor and NAT tissue. These findings suggested that normal-appearing prostate tissue could undergo genetic changes in response to or as a prelude to cancer.

Another relatively early study compared the mRNA expression profiles in breast cancer tissue with NAT and age-matched control tissue from breast reduction patients [8]. There were 105 DEGs between the NAT samples and the healthy controls, with around 80% of these also appearing as DEGs between the healthy controls and cancer tissue (and in the same direction, i.e., up- or

down-regulation). The authors concluded that global gene expression abnormalities exist in the normal epithelium of breast cancer patients that are also present in early cancers.

In fact, although the value of paired NAT and cancer-free samples has not yet been fully examined, reports suggest that mRNA and protein expression level changes in NAT may be more predictive of cancer relapse and survival than expression levels in tumor samples alone. Therefore, NAT may prove useful for predicting disease prognosis.

For example, using RNA extracted from histologically normal breast tissue from 107 patients, including 60 breast reduction patients and 47 cancer patients, whole transcriptome profiling identified a gene expression signature associated with wound response in the NAT tissue that was induced in response to breast cancer, and this signature was highly prognostic of breast cancer survival [15]. As another example, a recent study analyzing data in The Cancer Genome Atlas (TCGA) indicated that NAT samples are in general more informative regarding patient survival than tumors and that this is likely due to tumor microenvironment effects rather than through tumor cell contamination or field cancerization [18].Pathway analyses suggested that the TME may play an important role in cancer patient survival by boosting the activity of metabolism- and immune-related pathways in NAT. Similar studies include those on colorectal cancer [35,36] and breast cancer [38]. Another study, while not including cancer-free controls, did link the protein expression of the cancer-testis antigen, AKAP3, in NAT with poor prognosis in breast cancer [38].

A landmark 2017 study have provided further evidence for the intermediate status of NAT, for not just one but eight different types of cancer [10].For this study, the researchers combined and analyzed the transcriptome data from two open-access databases: the Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) project. The GTEx project includes the genomic profiles of thousands of tissue samples from deceased donors, including many individuals who did not have cancer, while the TCGA contains the genomic profiles of thousands of tumor samples and hundreds of NAT samples from multiple tissues.

In total, this study compared the molecular profiles of 6,500 tissue samples covering eight cancer types, including of the breast, liver, colon, and prostate [10].While there were unique signatures for each cancer, their analysis also revealed a common signature in NAT tissue for all eight cancers. This signature comprised a set of genes that were specifically over expressed in NAT tissues compared with both healthy tissues and tumors, and a strong association was found between this signature and TNF-α and TGF-β signaling pathways, hypoxia, and epithelial-to-mesenchymal transition (EMT).

Their findings suggest that, in contrast to the field cancerization theory (which implies that an evolutionary process forms the NAT phenotype prior to tumorigenesis) the tumor itself has an active role in shaping a unique, dynamic phenotype in the NAT [10]. In other words, interactions with the tumor and the TME may help shape the NAT microenvironment through the spread of pro-inflammatory signals from the tumor to its surroundings, which induce the signaling pathways responsible for the distinct NAT phenotype.

Thus, stromal changes in NAT represent an emerging hallmark of cancer that may be essential for tumorigenesis and/or tumor progression [10]. The author’s preliminary experiments with mice showing effective disruption of this interplay between tumors and NAT may, in the future, offer an important therapeutic strategy.

Epigenetic differences

For several decades, cancer was perceived mainly as a disease of the genome, predominantly caused by genetic mutations. However, epigenetic alterations are now known to be a universal feature of cancer, associated with almost every step of tumor development and progression [39]. Indeed, instead of epigenetic changes being merely a consequence of primary genetic alterations, there is increasing evidence for the concept of epigenetic drivers in cancer.

Although primary tumor cells harbor an array of cancer-specific genetic mutations, less than 0.01% of the primary cancer cells that enter circulation can metastasize [39]. It is now becoming evident that epigenetic changes play key role inconferring additional properties to primary cancer cells that contribute to the metastatic process. Metastasis is a dynamic, multi-step process with cancer cells exhibiting various phenotypic transitions-unlike genetic events, epigenetic changes are also dynamic.

"Driver mutations" are crucial genetic mutations that initiate tumorigenesis by providing a selective growth advantage to the cancer cell [40]. However, the definition of cancer drivers has recently been extended to include any alterations that contribute to any stage of tumor evolution and progression. This new definition includes and recognizes the contribution of epigenetic changes as potential driver events in cancer initiation and progression [39].

a) DNA methylation

The methylation of CpG dinucleotides is a stable and inheritable epigenetic modification [41].This epigenetic mechanism regulates genes involved in various fundamental biological processes, including cell differentiation and cell development. On the other hand, aberrant DNA methylation in cancer tissues relative to NAT has been shown to be associated with carcinogenesis. Indeed, promoter DNA methylation-mediated gene silencing is by far the most recognized epigenetic event in cancer, and hypermethylated regions are promising cancer biomarkers [41].

Few studies, however, have investigated epigenetic changes across healthy tissue, NAT, and cancer tissue. A study earlier in 2020 used data from the Gene Expression Omnibus (GEO) to develop an improved algorithm for detecting changes in DNA methylation between breast tissues from three groups: 1) healthy, no breast cancer; 2) NAT from breast cancer patients; and 3) breast cancer tissue [42].Intriguingly, they identified novel epigenetic field effects (i.e., where epigenetic alterations had accumulated in normal-appearing tissues in cancer patients) associated with breast cancer and progression. The authors noted that their method uncovered epigenetic effects that would not have been identified using traditional methods such as EWAS (epigenome-wide association studies). The findings of this study suggest that NAT tissue, in addition to exhibiting an "intermediate" state in terms of mutation frequency (as discussed in Section 2), may also exhibit an intermediate epigenetic state between that of cancerous tissue and normal tissue from a healthy individual.

DNA methylation is reversible and therefore, removal of methylation marks can lead to the activation of potential oncogenes [39]. However, the main focus for many years has been the hypermethylation of the promoters of tumor suppressor genes (leading to gene silencing), whereas hypomethylation-directed activation of oncogenes has been relatively less studied.

b) Chromatin structure

Compared with DNA methylation as an epigenetic phenomenon, much less work has been performed on histone modifications and other factors influencing chromatin structure in tumors versus NAT. The analysis of several different cancers has revealed the differential

expression of several chromatin remodelers (e.g., ARID1A, ARID1B, ARID2), insulator proteins (e.g., CTCF), Histone H3 subunit-related genes (ATRX, DAXX, H3F3A, HIST1H3B, HIST1H1C), histone methylation erasers (e.g., EZH2), and histone acetylation writers and erasers (genes that encode histone acetyltransferases) [39]. DNA methylation is also associated with histone modifications and the interplay of these epigenetic modifications is crucial in regulating the functioning of the genome by changing chromatin architecture.[41]Further, mismatch repair is a critical DNA repair mechanism involved in maintaining microsatellite stability, which, in turn, is associated with chromatin organization and recombination [43]. Further research is required to determine the chromatin status in NAT tissue relative to healthy and cancerous tissue.

c) Non-coding RNAs

Recent advances in sequencing technologies have revealed that <2% of all transcripts code for proteins. This has led to a paradigm shift and a rush of research into the noncoding transcriptome [24].

- ***MicroRNAs***

MicroRNAs (miRNAs) are a group of short noncoding RNAs approximately 22 nucleotides in length [44]. The primary function of miRNAsis the negative regulation of their target genes at the post-transcription level. Specifically, miRNA induces transcript degradation or inhibits protein translation through sequence-specific binding to the 3′UTR of its target mRNAs. To date, multiple miRNA have been identified as differentially expressed in various types of cancers, and there is accumulating evidence that miRNAs regulate a wide range of biologic processes in cancers such as proliferation, apoptosis, metastasis, chemosensitivity, cancer stem cell maintenance, and tumor development, through the over expression of oncogenic miRNAs and downregulation of tumor suppressor miRNAs [45]. MiRNAs have further been linked to dysregulated epithelial-mesenchymal transition (EMT) in cancers, such as the miRNA-mediated EMT in non-small cell lung cancer [46].

Most studies on miRNA in NAT have focused on comparisons of NAT with tumor tissues. MiRNAs particularly show promise as biomarkers for distinguishing between tumor and NAT and between various subgroups of tumors [47-49].

Some studies have, however, causally linked miRNA-mediated crosstalk between tumors and NAT and more distant sites with tumor progression and metastasis. For example, metastatic hepatocellular carcinoma (HCC) cells have been shown to convert normal fibroblasts into cancer-associated fibroblasts (CAFs) [50]. Highly metastatic HCC cells have an increased capacity for conversion compared with low-metastatic HCC cells, and this effect appears to be exerted through the secretion of miR-1247-3p from the HCC cells via extracellular vesicles. Moreover, high serum levels of miR-1247-3p correlate with lung metastasis in HCC patients.

As another example of this crosstalk but in the opposite direction (NAT to tumor), healthy epithelial cells are known to secrete exosomes containing several tumor-suppressive miRNAs such as mIR-143 [51]. When precancerous cells acquire resistance toward these anti-proliferative miRNAs or when normal cells can no longer supply an adequate amount of these miRNAs, this defense system eventually fails and this can lead to tumorigenesis.

Because miRNAs are considerably smaller than proteins, they could be introduced into NAT and tumor cells using the same techniques used for siRNA [44]. This has fostered research into miRNA delivery strategies for the treatment of cancer patients.

Long non-coding RNAs

Most of the genome is now known to encode long non-coding RNAs (lncRNAs), which are typically defined as RNA transcripts longer than 200 nucleotides with no protein-coding potential [24,52]. Many lncRNAs are expressed at low levels and display tissue- and cell type-specific expression patterns. They participate in processes ranging from transcriptional regulation and chromatin remodeling to the control of protein translation. Thousands of lncRNAs have been discovered, but only a few have been shown to play crucial biological roles and there are still many knowledge gaps.

Studies investigating lncRNAs in NAT have focused on comparisons of lncRNA levels in cancer relative to NAT [24]. Indeed, cancer-specific expression of certain lncRNAs relative to NAT first suggested that these molecules may play a role in tumorigenesis and cancer progression, and an increasing number of dysregulated lncRNAs have been shown to mediate cell-cycle control, proliferation, apoptosis, and metastasisin cancer. For example, chromatin modification, epigenetic regulation, alternative splicing, and translational control by lncRNAs such asMALAT1, HOTAIR, and TRE are well-established examples of lncRNA-mediated control of cell migration and invasion, EMT, and metastasis [53]. Importantly, lncRNAs can be selectively packaged into exosomes (Section 7), and exosomal lncRNAs are now known to play a central role in carcinogenesis, cancer progression, and chemoresistance [54]. These lncRNAs function as messengers in cell-to-cell communication, facilitating remodeling of the tumor microenvironment.

Besides lncRNAs, other less investigated non-coding RNA species such as small nucleolar RNAs (snoRNAs, scaRNA), tRNA-derived fragments, and piwi RNAs (piRNAs) also have potential for application as cancer biomarkers and therapeutic targets [49,55]. Further research is required to determine how these non-coding RNAs differ in NAT tissue relative to healthy and cancerous tissue.

Circular RNAs

Circular RNAs (circRNAs) are highly stable, long, non-coding RNAs that result from the non-canonical splicing of linear pre-mRNAs [56].They are implicated in the modulation of gene expression and, therefore, possess diverse biological functions. CircRNAs appear to exert their effect via activity as miRNA reservoirs (“sponges”) with many miRNA binding sites, thereby influencing the expression of the downstream targets of these miRNAs.

Based on the key role of circRNAs in regulating gene expression, dysregulated circRNA function is likely to play a role in the aberrant gene expression and biological behavior observed in cancer cells [56]. Notably, a negative correlation has been observed between global circular RNA abundance and cancer cell proliferation. For example, bioinformatics workflows applied to breast cancer and NAT samples revealed that NAT tissues with an estrogen receptor-positive (ER+) subtype have relatively higher numbers of circRNAs than tumor samples [57]. Notably, the number of circRNAs in the NAT of the ER+ subtype was inversely correlated to the risk-of-relapse proliferation.

Exosomes

There are three main types of signaling mechanisms involved in cell-to-cell communication: cell contact dependent signal transduction, signaling transduction mediated by soluble molecules, and exosomes. Exosomes are extracellular microvesicles that act as substance transport carriers to facilitate biological information exchange between adjacent and distant cells [58-60]. They assist in the regulation of the normal cellular microenvironment via the delivery of a range of biological molecules, including proteins, lipids, DNAs (mitochondrial and genomic), mRNA, long non-coding

RNAs, and microRNAs. Exosomes are found in the majority of, if not all, biological fluids, and are secreted by a variety of cells.

Tumor cells are known to release high quantities of exosomes [61]. These exosomes are information carriers, conveying molecular and genetic messages from tumor cells to NAT or to normal and other abnormal cells residing in close or distant sites via the bodily fluids. Upon contact with target cells, they alter the phenotypic and functional attributes of the recipients. In cancer, exosomes are involved in tumor angiogenesis, tumor metastasis, and drug and radiotherapy resistance. There is also evidence that exosomes serve an important role in tumorigenesis [62].Tumor cells can transfer their contents, such as oncogenes, to normal cells within NAT and more distant sites, leading to tumor invasion and metastasis. On the other hand, the exosomes of normal cells in NAT can inhibit the proliferation of tumor cells by transferring tumor suppressor genes into the cancerous cells, allowing these genes to block the corresponding signaling pathways. Exosomes can also induce specific immune-based antitumor effects [62].

Exosomes are of potential interest as noninvasive biomarkers of cancer through liquid biopsy, and their role in inhibiting host antitumor responses and mediating drug resistance is important for cancer therapy [61]. Exosomes also have potential utility as non-immunogenic vehicles for drug delivery to target cells [63].

Differences at the metabolomic level

Several studies have compared cancerous tissue to NAT tissue to identify differences in their metabolome (i.e., the types and quantities of the different metabolites present). However, these studies have typically focused on finding metabolomic signatures that differentiate between cancer types and between cancer and healthy controls [64], rather than illuminating the metabolomic status of NAT tissue relative to cancer-free tissue.

One study looked at the metabolic signatures in the serum of hepatocellular carcinoma (HCC) patients and compared these to NAT tissue and distant normal tissue (DNT) isolated from the same patients [65]. They found a significant overlap between the NAT and DNT samples, and therefore, excluded NAT from further analyses.

Another study used nuclear magnetic resonance (NMR) to compare the metabolomic signature of tissues from patients with Barrett's esophagus (BO) and esophageal adenocarcinoma (EAC) along with NAT tissue where possible, as well as controls from healthy patients who underwent endoscopy for dyspeptic symptoms but the samples were later found to be histologically benign [66]. Barrett's patients are known to be at increased risk for EAC. A comparison of NAT tissue proximal to EAC versus normal tissue from the controls identified a distinct signature of eight metabolites that were significantly altered. This signature may have potential diagnostic value for detecting tissue from which neoplasms could subsequently arise, and thus, it may be useful for identifying patients at high risk of developing EAC.

An interesting recent breast cancer study specifically compared data from normal and NAT tissue from the Normal Breast Study (NBS) in terms of metabolomics, gene expression, and histology [67]. Unsupervised clustering analysis identified two metabolomics-derived subtypes. The metabolite differences between the two clusters suggested enrichment of pathways involved in lipid metabolism, cell growth and proliferation, and migration in NAT. Furthermore, there were differences between the clusters based on the subject’s body mass index, adipose proportion, and genomic subtype. Further

research is needed to understand the potential role of these factors in breast cancer.

- ***Calcium signaling***

Deregulation of the calcium signal is often deleterious and has been linked to each of the cancer hallmarks [68]. Notably, there have been several, specific changes observed in aspects of calcium signaling between tumor and NAT tissues, such as the nature of Ca^{2+} influx or the rate of recovery of [Ca^{2+}] after stimulation, rather than simply changes in intra- and extra-cellular levels of calcium. Tightly controlled regulation of the calcium signal is essential for appropriate cellular functioning, as evidenced by the role of calcium in processes such as cell proliferation, gene transcription, and cell death.

Differences in mitochondria

Mitochondria, known as the energy powerhouses of the cell, represent a key intracellular signaling hub [69]. These organelles are emerging as important determinants of several aspects of cancer development and progression, including metabolic reprogramming, acquisition of metastatic capability, and response to chemotherapeutic drugs [70].

The majority of cancer cells harbor genetic and epigenetic aberrations in the mitochondrial genome (mtDNA), leading to mitochondrial dysfunction [69]. However, these alterations do not shut down mitochondrial functionality. Instead, they promote rewiring of the cancer cells through mitochondria-to-nucleus signaling that results in changes in the transcription and/or activity of cancer-related genes and signaling pathways. Their multiple functions allow mitochondria to sense cellular stress and promote cell adaptation to challenging microenvironments, thereby conferring a high degree of plasticity to tumor cells for growth and survival.

In a prescient study in 2005, somatic mitochondrial DNA mutations in three tissue types, namely tumor, adjacent benign (NAT), and distant benign, were investigated in prostate cancer [71]. Needle biopsy tissue from individuals referred for prostate biopsy, yet histologically benign (symptomatic benign), were also included, as well as blood samples from the prostate cancer patients. In contrast to both control groups within patient (blood) and among patient (symptomatic benign), all of the other tissue types harbored significantly different mtDNA mutation profiles. The distribution pattern and load of somatic mutations between the symptomatic benign control and the various malignant groups showed an accelerating trend, suggesting that these mutations may be an early indicator of malignant transformation in prostate tissue and occur well before histological changes. They further concluded that the use of benign tissue as a normal control, based on histological criteria alone, is inappropriate. Other studies have indicated a similar increasing trend in mtDNA mutations between healthy, NAT, and tumor tissues [72].

Genetic and epigenetic aberrations of the nuclear genome can also affect mitochondrial functioning through, e.g., reduction/depletion of mtDNA. A 2015 study on colorectal cancer (CRC) analyzed the mtDNA copy number invarious CRC sample types [73]. They found that while normal tissue from individuals without CRC did not have a significantly different mtDNA copy number from that of CRC NAT tissue or cancer tissue, a significantly higher mtDNA copy number was observed in recurrent CRC patients compared to primary CRC patients.

CONCLUSION

The tissue adjacent to the malignant tumors was long considered to be normal on the bases of morphology as evaluated by routine microscopy. Extensive omic-analysis has clearly demonstrated numerous changes in the tissue next to the tumors up to one centimeter. There is evidence of significant interactions between the tumor cells and the adjacent morphologically normal cells. As

described, multiple studies indicated that the NAT is an intermediate state between the normal and malignant tumors. These are two entities which are not independent of each other. There is extensive interaction and exchanges resulting in the NAT acquiring some of the features of malignancy. A detailed understanding of these interactions and the pathways involved could open new avenues for therapeutic intervention and may also provide important prognostic information.

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