Histopathology

Histopathology 2023, **82**, 162–169. DOI: 10.1111/his.14794

REVIEW

Next-generation sequencing in breast pathology: real impact on routine practice over a decade since its introduction

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(2023) *Histopathology* **82**, 162–169. https://doi.org/10.1111/his.14794

Next-generation sequencing in breast pathology: real impact on routine practice over a decade since its introduction

The diagnosis, histomolecular classes of breast cancers (luminal A, luminal B, HER2-enriched, and basal-like), and accurate prediction of prognosis are commonly determined using morphological and phenotypical analyses in clinical practice worldwide. Therapeutic strategies are mostly based on the disease stage and molecular subclasses of breast cancer. Targeted therapies, such as anti-HER2s, poly-ADP ribose polymerase inhibitors or, to a lesser extent, phosphatidylinositol 3 kinase inhibitors, have substantially improved breast cancer patient prognosis over the past decades. Human epidermal growth factor receptor 2 (HER2) overexpression is widely determined based on immunohistochemistry, while next-generation sequencing (NGS) is currently employed to assess the presence of molecular alterations, including *breast cancer gene 1* (*BRCA1) and 2* or *phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA)* mutations, which are targets of these new approved therapies. In addition, next-generation sequencing (NGS) can aid the pathologist in challenging situations, such as a diagnostic workup for a metastatic carcinoma in lymph nodes of unknown origin, differential diagnosis of spindle cell tumourtumor in the breast between metaplastic carcinoma, malignant PT and sarcoma, o, as well as determining relatedness between primary breast cancers and recurrences. NGS offers a powerful tool that enables the pathologist to combine morphological analyses together with molecular alterations in challenging diagnostic situations.

Keywords: *BRCA1*, *BRCA2*, breast, breast cancer, metaplastic carcinomas, next-generation sequencing, *HER2*, phyllode tumours, *PIK3CA*

Introduction

In breast cancer patients, massive parallel sequencing or next-generation sequencing (NGS) is most often used for identifying targetable alterations in the setting of advanced or metastatic disease. In a recent issue of the *Journal of Clinical Oncology*, a panel of experts reported the American Society of Clinical Oncology (ASCO) provisional clinical opinion concerning somatic genomic testing in patients with advanced or metastatic cancer, recognising that genomic sequencing may equally provide diagnostic or prognostic information.[1]

Over the years, cDNA microarray-based gene expression profiling, gene expression-based prognostic signatures, targeted Sanger sequencing, pan-genomic analyses [comparative genomic hybridisation (CGH)] and NGS have substantially broadened our

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Table 1. Surrogate markers and histomolecular classes of breast cancers adapted from AJCC (8th edition) and ESMO guidelines[48]

Intrinsic subtypes	Clinicopathological surrogate definition	Prognosis risk category based on multiparameter molecular marker if available
Luminal A	ER- and PR-positive and high; obvious low proliferation rate (low KI67, low mitotic count generally histopronostic grades 1 or 2) HER2-negative	'Favourable prognosis'
Luminal B Luminal B HER2–	Lower ER/PR than in luminal A, with high proliferation rate (high *Ki67, high mitotic count and generally histopronostic grade 3) HER2-negative	'Unfavourable prognosis'
Luminal B HER2+	HER2-positive ER-positive Any PR Any Ki67, generally histopronostic grade 3	
HER2-enriched	ER- and PR-negative HER2-positive	
Basal-like	ER- and PR-negative HER2-negative	

ER, oestrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; AJCC, American Joint Committee on Cancer; ESMO, European Society of Medical Oncology.
*Generally considered 'high' above 20% (typical median value in hormone receptor-positive cancers); definitely considered 'low' if lower or equal than 10%, and 'high' if higher or equal than 30%.

knowledge regarding breast cancer diversity and heterogeneity, thereby dramatically improving breast cancer classification.[2] Currently, it is widely acknowledged that breast cancers are heterogeneous entities that are associated with morphological and molecular specificities and different prognoses.

Treatment strategies for breast cancer patients are determined through the integration of clinical and pathological disease stages, histological types and histomolecular characteristics. Morphological analysis of haematoxylin and eosin (H&E)-stained tissue sections and immunohistochemistry (IHC) are the necessary and powerful techniques applied to report the diagnosis of breast cancer and determine histomolecular subgroups, including luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) luminal B, HER2-enriched or basal-like subtypes. The eighth edition of the American Joint Committee on Cancer (AJCC) staging manual published in 2018, as well as the European Society of Medical Oncology (ESMO) and National Comprehensive Cancer Network (NCCN) guidelines, have provided definitions of these four classes using surrogate markers determined by IHC for oestrogen receptor (ER), progesterone receptor (PR), HER2 and Ki-67 combined with histological grade and mitotic index (Table 1).

Breast cancer is the most common cancer in women worldwide. Taking into account socio-economic differences across countries, it is essential that international guidelines for therapeutic decision-making are based on affordable and widely used diagnostic tests.

This review is focused on situations where NGS typically plays a role in medical practice for clinical management, as well as for diagnostic purposes. After a short outline of different available NGS technologies, four clinical situations are discussed in further detail:

- identification of 'druggable' molecular targets for early, advanced and metastatic breast cancer patients;
- diagnostic work-up of axillary lymph node metastases of carcinomas of unknown origin;
- differential diagnosis between metaplastic carcinoma, malignant phyllodes tumours (PT) and other spindle cell tumours of the breast; and
- relationship between primary breast cancer and recurrence.

Genetic testing technologies [gene panels, whole-exome sequencing (WES), RNA-seq and whole-genome sequencing (WGS)]

Since the early 2000s, the development of NGS and gradual decrease of its cost have enabled this

technique to be implemented in a hospital setting to assist with routine diagnosis and help to discover new therapeutic targets.[3] Private companies similarly provide NGS services for clinical purposes. Assay selection and data interpretation may vary according to local practice. Ideally, NGS results are now being discussed at molecular tumour boards where medical oncologists, pathologists, geneticists and bioinformaticians consolidate the integration of NGS with clinical and pathological data to accurately determine the best therapeutic option for the patient. It is crucial to keep in mind that health systems differ worldwide, and in some countries reimbursement of NGS tests are still pending.

NGS technologies allow for massively parallel sequencing of millions of deoxyribonucleic acid (DNA) molecules, having thus dramatically increased data throughput. Second-generation approaches, which are most currently used in routine diagnostic laboratories, are based on the sequencing of previously fragmented, ligated and amplified DNA molecules, called 'short reads'. Once sequenced, the reads are bioinformatically reassembled on a reference sequence in order to detect any variations.[4] Sequencing of short reads is particularly suitable for DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissues that are already partially degraded.

NGS enables sequencing of gene panels, WES and WGS, all of which provide valuable insights into the field of oncology. NGS allows for detecting point mutations, small base insertions and deletions, copy number variations and structural rearrangements.

Current sequencers can sequence up to millions or even billions of reads per sample, rendering it possible to analyse genes of interest with high depth of coverage, which corresponds to the number of reads at a given position.[5] Great depth is particularly necessary to detect subclonal tumour mutations or mutations in samples with low tumour cellularity. The choice of platform to be used is a compromise between the number of genes or genomic regions to be covered and the depth desired to detect mutations with a low variant allele frequency.

Ribonucleic acid (RNA) can be analysed using the same sequencing platforms by performing reverse transcription (RT) of RNA molecules into complementary DNA molecules prior to sequencing. Sequencing messenger RNAs can provide additional information regarding differential expression, gene fusions and alternative splicing. In addition, some mutations can similarly be inferred from RNA sequencing data.

A small panel of a few dozen genes is particularly suitable for analysing the main known and targetable oncogenes in a fast and cost-effective way. The tumour DNA to be analysed can be extracted from FFPE,[6] frozen tissues or fluids such as blood (i.e. 'liquid biopsies').

A larger panel of hundreds of genes can be employed to search for new therapeutic targets, detect new biomarkers for disease monitoring and test for clinical trial eligibility based on newly detected molecular alterations. In addition, this larger-scale sequencing allows for estimating tumour mutational burden (TMB) and microsatellite instability (MSI) status using bioinformatics tools. Nevertheless, genome-wide structural variants are not detected in the gene panel settings, as only specific genes are covered.

Last, but not least, low-coverage whole genome sequencing with approximately 1× of coverage can be applied to detect copy number alterations and specify homologous recombination deficiency [i.e. 'shallow homologous recombination deficiency (HRD)' genomic test] using dedicated specific algorithms.[7] In the near future this technique may become a very useful and cost-effective way to identify patients that could benefit from poly-ADP ribose polymerase inhibitors (PARPi). Moreover, this technique generates small-volume, storable data and is suitable for FFPE samples.

Identifying 'druggable' molecular targets for early stage, advanced and metastatic breast cancer patients

During the past 10 years, using NGS was mainly limited to clinical trials. More recently, through a collaborative effort, ESMO has proposed a scale for clinical actionability of molecular targets (ESCAT), which ranks molecular targets based on evidence supporting their value as druggable targets.[8]

Genomic alterations are ranked as targets for precision cancer care according to their clinical benefit for patients. Alterations with level I evidence are those for which alteration drug-matching was proved to be associated with improved outcome in clinical trials. As of 2021, only three alterations in breast cancer were ranked as level IA (i.e. having shown a clinical benefit in prospective randomised trials dedicated to breast cancer): *erb-b2 receptor tyrosine kinase 2* (*ERBB2*) amplification; *PIK3CA* mutations; and *BRCA1* and *BRCA2* germline mutations.[8]

ESMO guidelines for patients with newly diagnosed recurrent or metastatic breast cancer recommend a biopsy of the recurrent/metastatic disease, if feasible, in safe and acceptable conditions for the patient in order to reassess the phenotype of the metastatic

disease (ER, PR and HER2). Determining *BRCA1* or *BRCA2* germline status is similarly recommended, as *BRCA1* and *BRCA2* mutations are targetable using PARP inhibitors. Screening for these mutations is carried out at the metastatic stage, as the clinical BROCADE-3 trial results have shown a clear veliparib benefit in this setting.[9,10] It is also conducted at early-stage breast cancer (triple-negative or luminal), as the OlympiA trial results revealed improved 3-year invasive disease-free survival (3yIDFS) rates from 77.1 to 85.9% via targeting *BRCA1–BRCA2* mutations with adjuvant olaparib.[11]

Based on local practice, genetic counselling is offered rapidly or in parallel with the germline or somatic analysis should a *BRCA1/2* variant have been identified.

NGS has also allowed for investigating treatment-resistant breast cancer, thereby unravelling regarding 40 recurrent driver alterations in this type of disease.[12] The response to some targeted treatments has been validated in several clinical trials. For HR+ tumours, genomic profiling has been able to identify *Pi3Kca* or *AKT* mutations underpinning treatment resistance.[13] This has led to a change in the standard of care, given that mammalian target of rapamycin (mTOR) and PI3K inhibitors were approved for use in combination with endocrine therapies in this setting.[14] Alpelisib, an alpha-specific PI3K inhibitor, combined with endocrine therapy in *Pi3KCA*-mutated breast cancer patients, significantly prolonged progression-free survival (PFS), as demonstrated in the SOLAR-1 trial.[15] *Pi3KCA* hot-spot mutations have been classified as level IA mutations in clinical practice, according to the ESMO scale for clinical actionability of molecular targets (ESCAT).

Monitoring breast cancer progression by means of circulating tumour DNA (ctDNA) represents another valuable opportunity for breast cancer patient care. *Oestrogen receptor 1 gene* (*ESR1*) mutations are acquired upon aromatase inhibitor treatment, being subclonal in the tumour. Thus, their detection in ctDNA is more accurate and can easily be performed using droplet digital polymerase chain reaction (PCR).[16] In the PADA-1 trial, the presence of *ESR1* mutations in ctDNA was tested at baseline, while being repeated upon first-line treatment for metastatic, ER+ HER2– breast cancer with the association of palbociclib and aromatase inhibitors.[17] Patients who had been randomised to a switch from an aromatase inhibitor to fulvestrant upon early identification of an *ESR1* mutation experienced a doubling in median PFS. This trial has further emphasised the role of liquid biopsy and ctDNA in patient treatment monitoring. *ESR1* mutations are currently classified as ESCAT evidence of level IB (biomarkers that predict response or resistance to therapies for a specific type of tumour); in the near future these mutations are likely to become a standard of care, in combination with surveillance based on functional imaging.

Two other molecular alterations have been recognised as predictive markers with ESCAT evidence level IC in breast cancers: microsatellite instability (MSI) for immune check-point inhibitors and identification of *neurotrophic tyrosine receptor kinase* (*NTRK*) fusion for larotrectinib or entrectinib, both being tyrosine kinase (TRK) inhibitors. Nevertheless, the prevalence of these alterations in breast cancer patients appears to be somewhat low, being observed in fewer than 5% of cases.

While the best testing strategy is still a matter of debate, IHC represents a powerful tool that could allow for selecting cases for molecular testing in order to confirm mismatch repair (MMR) status or *NTRK* fusions. Recently, published recommendations proposed to use IHC as a first-line test, which should be followed by reverse transcription–polymerase chain reaction (RT–PCR) for cases that are positive for the NTRK protein (nuclear expression in breast cancers) to confirm the presence of a fusion transcript.[18] For MSI, the commonly applied diagnostic procedure is molecular screening.[19]

Diagnostic work-up of axillary lymph node metastases of carcinomas of unknown origin

Carcinoma of unknown primary (CUP) is a clinical entity that refers to metastatic disease of occult cancer. It represents 2–3% of metastatic cancers diagnosed every year.[20] More than half the patients display disseminated disease, and only 15% exhibit unique axillary lymph node location.[21] Biopsy of the metastasis is an essential step to determine the potential origin of the cancer and direct treatment choices. Extensive IHC analysis should be performed in order to narrow the range of diagnostic possibilities using antibodies directed against proteins, such as CK7, CK20, CK8/18, mammaglobin, GCDFP-15, TRPS1,[22] HER2, ER and PR.[23] However, IHC has occasionally failed to offer an unequivocal diagnosis.[24]

Gene expression profiling, including unsupervised clustering, can substantially help in predicting the tissue of origin,[25] which could enable determination of the origin of up to 75% CUP tumours.[26]

RNA sequencing, together with a classifier tool using deep learning, was developed at the Institut

Curie in an effort to identify the origin of metastatic cancers. We tend to use this tool in clinical practice when a frozen specimen of the metastatic disease is available after extensive IHC analysis has failed to clearly identify its origin.[27]

Differential diagnosis between metaplastic carcinoma, malignant phyllodes tumour and other spindle cell tumours of the breast

Metaplastic breast carcinoma (MBC) is a rare special type of breast cancer while being part of a heterogeneous group of tumours. Malignant epithelial cells in metaplastic carcinoma differentiate into squamous, mesenchymal element or spindle-shaped cells. MBC can be monomorphic or biphasic, i.e. forming a mixed tumour with two or more components, such as an adenocarcinoma component admixed with squamous or chondroid elements. Numerous MBCs are of high histological grade and display a triple-negative phenotype.[28]

PT are biphasic fibroepithelial neoplasms with increased stromal cellularity. They are classified as benign, borderline or malignant tumours based on microscopic criteria such as mitotic index, the degree of stromal overgrowth, atypia, hypercellularity and infiltrative borders.[29]

Due to these histological features, monophasic high-grade spindle cell MBC can be misdiagnosed as another high-grade spindle-cell tumour of the breast and particularly as a malignant PT if the epithelial component is not visualised in the diagnostic sample. In difficult cases with overlapping morphologies, molecular testing can help to orientate the correct diagnosis.[30]

To illustrate, 53% of MBCs have been shown to harbour *TP53* mutations.[28] Notably, *TP53* alterations were identified in both carcinomatous and sarcomatous components of MBCs, supporting the monoclonal origin of these components as being morphologically distinct. Conversely, mutations in *TP53* have been reported in borderline and malignant PT at a much lower frequency, ranging from 4%[31] to 10%.[32]

Epidermal growth factor receptor (EGFR) amplification has been reported in up to 80% of squamous MBC, whereas no activating mutations in *EGFR* were detected.[33–35] Conversely, mutations in *EGFR* have been observed in rare borderline and malignant PT,[36] while *EGFR* amplifications were detected in only 2–16% of all PT and nearly 20% of malignant PT.[37] Taken together, concerning the prevalence of *EGFR* molecular alterations, activating mutations are more correlated with malignant PT, while *EGFR* amplifications are correlated with MBC. Thus, these molecular findings are probably instrumental in the differential diagnosis between MBC and malignant PT.

Telomerase reverse transcriptase (*TERT*) alterations have been reported in up to 25% of MBC, being mainly *TERT* promoter mutations in spindle-cell and squamous MBC, and less commonly in MBC exhibiting a predominant chondroid component.[38,39] In the series published by da Silva *et al.*, *TERT* alterations were negatively correlated with *TP53* mutations while being positively associated with *PIK3CA* mutations.[38]

In PT, a hot-spot *TERT* promoter mutation (−124 C > T) was found in 52% and *TERT* amplification in only 4% of cases, respectively.[36] While these alterations were restricted to the mesenchymal component, they were significantly more common in borderline and malignant PT than in benign PT. *TERT* mutations were observed in 7% of fibroadenomas in one study,[40] with no *TERT* alterations detected in a series of 100 fibroadenomas analysed by Piscuoglio *et al.*[36] Furthermore, *TERT* promoter mutations were observed in a series of primary sarcomas of the breast (non-angiosarcomas), whereas no *TERT* alteration was detected in angiosarcoma of the breast.[41] Altogether, in the context of those observations, *TERT* promoter mutations may be useful in the differential diagnosis: (1) between fibroadenoma and PT; (2) between angiosarcoma and malignant PT; and (3) between angiosarcoma and another type of primary breast sarcoma. In addition, *TERT* somatic alterations have been demonstrated in 13% of adenomyo-epitheliomas; these are tumours of uncertain malignant potential that may progress to spindle cell MBC.[42]

PIK3CA, phosphoinositide-3-kinase regulatory subunit 1 (*PIK3R1*) and *PTEN* mutations have proved to be an early event in MBC being reported in 23–70% of cases; they appear to be more common in MBC with spindle cell or squamous metaplasia.[43] In contrast, chondroid-predominant MBC mainly lacks *PIK3CA* and *TERT* promoter mutations.[28,38,39]

Mediator complex subunit 12 (*MED12*) exon 2 mutations were found in fibroadenomas and in all histological grades of PT. Microdissection analysis confirmed *MED12* mutations to be stroma-confined in these fibroepithelial lesions,[37,40] and malignant PT were significantly less likely to harbour *MED12* mutations than fibroadenomas and benign or borderline PT.[44]

MED12 mutations were additionally observed in some primary breast (non-angio) sarcomas (67%)

Table 2. Most frequent mutations observed in phyllode tumours, spindle cell metaplastic breast carcinomas, angiosarcomas and other sarcomas of the breast

	MED12 mutation	TERT mutation	TERT amplification	RARA mutation	EGFR mutation	EGFR amplification	PIK3CA mutation	TP53 mutation
PT	+	+	+/−	+	+	−	+/−	+
SCMBC	−	−/+	+	−	−	+	+	+
AS	−	−	−					
Other sarcomas	+	+	+					

AS, angiosarcoma; PT, phyllodes tumour; SCMBC, spindle cell metaplastic breast carcinoma.

and in one MBC arising in the background of a PT.[34,45] Thus, the identification of *MED12* mutations in a spindle cell tumour of the breast is in favour of a PT diagnosis.

Retinoic acid receptor alpha (*RARA*) mutations were reported in 23% of PT[32]; to our knowledge, such mutations have not been reported in MBC to date.

In summary, high-grade spindle cell breast cancer is a heterogeneous group that encompasses mainly MBCs without epithelial differentiation, malignant PT and sarcomas. Indeed, many MBCs have lost their 'morphological' differentiation on H&E staining; they thus appear to be spindle cells. Nevertheless, they still display epithelial differentiation on IHC staining (pan-cytokeratin). Differential diagnosis is based upon morphological features, extensive IHC panel and NGS. Molecular profiles of MBC and malignant PT display different but also overlapping molecular alterations (although the latter at different frequencies).

Therefore, while NGS may be instrumental in distinguishing the two entities, this technology should be used cautiously. The final diagnosis also relies upon integration of the clinical context, histo-morphology, phenotype and molecular lesion portrait. It is essential to note that some tumours may harbour a specific molecular alteration, while others may display one or more non-specific molecular alterations; however, it is the combination of all findings that should help to establish an accurate diagnosis (Table 2).

Determining relatedness between a primary breast cancer and late recurrence

Several clinical situations can be challenging in routine practice, such as differentiating late relapse after early-stage disease from *de-novo* metastatic disease; explaining discordant clinical response of a multifocal disease; and understanding the cause of treatment resistance.

NGS allows for determination of the molecular profile of primary breast cancer while comparing it to the relapsed' profile. Metastases usually share at least some of the molecular alterations present in the primary breast cancer, whereas they reveal a high heterogeneous genomic profile, especially in luminal A and in late metastases.[46] Metastatic disease also acquires predominant driver mutations that already pre-existed in the primary tumour while having expanded under treatment pressure, including *ERS1* and mitogen-activated protein kinase (MAPK) pathway alterations [*EGFR*, *ERB2*, *RAS*, *neurofibromin 1* (*NF1*), etc.].[47]

In addition to the obvious interest of identifying acquired therapeutic targets upon disease progression, shared driver mutations or shared breaking points of chromosomal copy number alterations between primary and recurrent samples probably help to confirm filiation between the two tumours.[48]

Conclusions

After more than a decade since the implementation of NGS, these assays are mainly used to search for theranostic markers. Only two variants are currently recognised as theranostic markers with evidence level IA worldwide for breast cancer patients, including germline *BRCA1* and *BRCA2* variants for the indication of PARP inhibitors in triple-negative or luminal breast cancer patients, as well as *PIK3CA* mutations, knowing that many, but not all, countries have so far approved the use of PI3K inhibitors.

NGS assays are similarly helpful when pathologists are faced with difficult diagnoses: RNA sequencing and clustering combined with extensive IHC profiling of metastatic carcinomas of unknown origin represent valuable tools for pathologists. Identifying genomic alterations may similarly help to confirm a diagnostic hypothesis, knowing that the mutation rates in breast tumours are now available in public databases such as the cBioPortal (cBioPortal for Cancer Genomics). The challenging differential diagnosis between a grade 3 PT and a spindle cell metaplastic carcinoma

represents the most common situation in which NGS (gene panel) is being employed in our cancer centre for diagnostic purposes. The presence of *MED12*, *RARA* and, to a lesser extent, *TERT* mutations, can help to support the PT diagnosis, as can a poor phenotype. Conversely, the presence of a *TP53* mutation associated with a *PIK3CA* mutation in tumours expressing low keratin or p63 levels points towards the diagnosis of a spindle cell metaplastic carcinoma.

Despite the above-mentioned NGS applications in breast cancer, IHC continues to play a pivotal role in patient management. This technology appears to become even more critical with the recent use of antibody drug conjugates (ADC) in clinical practice, which represents a tremendous breakthrough in breast cancer patient treatment. The immune checkpoint inhibitor companion test is so far the programmed death ligand 1 (PD-L1) status determined by IHC in the metastatic setting. Taken together, integration of IHC and NGS results allows for a robust and personalised characterisation of breast cancers in today's routine pathology practice, which will undoubtedly remain critical in the near future.

Acknowledgements

The authors thank Dr Samia Melaabi for her assistance in preparing the manuscript and Dr Gabrielle Cremer from Cremer Consulting SA for editing the manuscript's English content.

Conflicts of interest

A.V.S. has received honoraria for lectures and research grants from Astra-Zeneca and Ibex, but has no other conflict of interest to declare in relation with this review's topic. The other authors have no conflicts of interest to declare.

Data availability statement

Data sharing is not applicable to this article, as no new data were created or analysed for this review.

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