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Review

Genetic aberrations as the targets of oncology research: Involvement of paraffin-embedded tissues

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Summary. Cancer is a complex and heterogeneous group of diseases which have been generally classified by their clinical and histopathological features. The genomes of cancer cells are altered by diverse mechanisms and these genetic aberrations lead to a variety of pathological changes. A number of technological advances have allowed us to analyze the cancer genome by various '-omics' techniques, and have accelerated the exploration for the primary genetic aberrations that drive cancer. The state-of-the-art technologies that have developed over the past few decades have enabled researchers to catalogue these genetic aberrations in detail. These aberrations include changes in gene structure and the copy number, mutation, and modification of DNA. Simultaneously, there have been significant achievements in the translation of the genomic discoveries "from the bench to the bed", which have provided valuable contributions to the progress in cancer therapy. One technology that has been central to these research efforts has been the histopathology of cancer specimens, particularly the use of formalin-fixed, paraffin-embedded tissues. In this overview, we consider the development of oncology research from the past to current efforts, and highlight the roles of histopathology and paraffin-embedded tissues in these efforts.

Key words: Paraffin-embedded Tissues, Genetics, Oncology, Histopathology

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1. Introduction

Cancers have been classified by their histopathological features, and these classification schemes have been standardized world-wide in the "WHO classification" system, comprised of ten fascicles of series. At the molecular level, human cancer cells are characterized by numerous chromosomal and nucleotide alterations which include irreversible aberrations in the DNA structure and sequence, and changes in gene or chromosome number. DNA can also undergo reversible alterations, such as epigenetic modifications in the gene and associated histones. In recent years, cancer genomics has seen significant progress due to the use of genome-wide, high-throughput platforms. These technologies supply multidimensional genomic data that can rapidly identify multiple changes in each cancer type and help to delineate the differences between individual cancers. Both reversible and irreversible changes in DNA alter the transcription and translation of a diverse range of genes, indirectly alter post-translational modifications and, ultimately, changes protein-mediated cellular functions (Chin and Gray, 2008). Cancer research was driven by elucidation of these oncogenic mechanisms through the discovery of "critical molecules", several of which have been clearly identified. A novel concept, "oncogene addiction", has played a key role in accelerating the path "from the bench to the bed" through such "critical molecules" (Weinstein, 2002). In addition, the concept of "oncogenic shock", i.e. the selective loss of survival signals by oncoprotein inactivation, has provided further insights into the potential for cancer treatment (Tedford

et al., 2009).

These developments in cancer research, and the identification of "critical molecules" and their aberrations, have changed some approaches to therapy, as they now allow physicians to stratify patients by the so-called "molecularly targeted approach". This approach involves the detection of specifically defined targets to assess whether a patient will benefit from a particular chemotherapeutic agent. This approach has been validated by the success of several molecularly targeted agents: Imatinib for chronic myelogenous leukemia (CML) with bcr-abl fusion gene and for gastrointestinal stromal tumors (GIST) with mutated *c-KIT*, gefitinib for non-small cell lung carcinoma (NSCLC) with mutated *Epidermal Growth Factor Receptor* (*EGFR*), trastuzumab or lapatinib for breast carcinomas overexpressing *human-EGFR2* (*HER2*), and rituximab for B-cell lymphomas overexpressing CD20 (Jazirehi and Bonavida, 2005; Siddiqui and Scott, 2007; Chin and Gray, 2008). These clinical successes have paved the way for the further design of drugs targeted to a relevant molecule, and more than 500 agents have been developed (Fabbro et al., 2012). Today, "individualized cancer therapy" has come of age.

Identification of the primary molecular aberration responsible for particular cancers is a conceptually simple, but reliable strategy to find the Achilles' heel of each cancer. In this new era of molecularly targeted therapies, however, treatment options are still dependent on the accurate "histopathological" profiling of each cancer (Chapter 2.1). Therefore, histopathology is one of the most important tools to dissect the character of cancer. Moreover, examination of the tissues embedded in paraffin is a relatively easy and accurate screening tool for determining the molecular profiles of various cancer types.

Herein, we describe past landmarks in histopathology in oncology research and discuss the possible involvement and roles of this method in the future, with emphasis on the utilization of paraffin-embedded tissues.

2. History of oncology research and histopathology

2.1. Histopathology as a cancer profile

In each organ, cancers are classified according to the WHO histological classification and this classification generally correlates with biological behavior, therapeutic response and prognosis. Therefore, histopathological features represent a major component of the cancer profile, and indeed, treatment decisions are often made based on the histopathological diagnosis in most types of cancers. For example, in the standard treatment for ovarian cancers, serous carcinomas are sensitive to platinum-based chemotherapy, while clear cell or mucinous carcinomas show chemo-resistance, resulting in poor prognosis (Takano et al., 2012). However, these conventional prognostic algorithms have not sufficiently adapted to the biological diversity of cancer, even within the same histological type, or to perform sufficient guidance for effective individualized therapy. Therefore, in breast carcinomas, current treatment guidelines combine traditional prognostic factors (TNM and Stage) with estrogen receptor, progesterone receptor, HER2 expression status, and in some cases, gene expression signatures (Chapter 4), which classify these cancers into several molecular subtypes (Perou et al., 2000; Sorlie et al., 2001; Blok et al., 2013).

2.2. Exploration of the specific cancer profile

In parallel with conventional histopathology, the trial for the identification of cancer profiles has historically emerged since the 1980s from an analysis of gross chromosomal rearrangements, allelic gains and deletions (Smolen et al., 2006). Most of the chromosomal alterations are translocations, which are still newly being found. Translocations often lead to fusion transcripts, and their identification not only provides novel diagnostic criteria, but also provides the basis for the development of new therapeutic strategies aiming at blocking aberrant activity of the chimeric protein. A typical example is the translocation that results in the formation of bcr-abl, a fusion gene found in 85% of CML (Jabbour and Kantarjian, 2012). Other known fusion genes (Table 1) include: translocations of t(11;22) or t(21;22) corresponding to the *EWSR1-Fli-1* or *EWSR1-ERG*, respectively, in Ewing's sarcoma, translocation of t(18, X) corresponding to SS18-SSX1/2 fusion in synovial sarcoma and the recently identified chromosome inversion inv(2)(p21;p23) which causes the *EML4–ALK* fusion in adenocarcinoma of the lung (Sorensen et al., 1994; de Alava, 2007; Soda et al., 2007; Cantile et al., 2013). In addition to chromosomal translocations or rearrangements, genetic analyses have identified a variety of gene amplifications and mutations,

Table 1. Chromosomal abnormalities in well-known tumors.

Fusion Gene	Chromosomal Translocation	Tumor
BCR-ABL	t(9;22)(q34;q11).	Chronic Myelogenous Leukemia (Jabbour and Kantarjian, 2012)
EWSR1-Fli-1	t(11 22)(q24; q12)	Ewing Sarcoma (Cantile et al., 2013)
EWSR1-ERG	t(21 22)(q22; q12)	Ewing Sarcoma (Sorensen et al., 1994),
SS18-SSX1/2	t(X 18)(p112; q112)	Synovial Sarcoma (de Alava, 2007)
EML-ALK	inv(2)(p21;p23)	Lung adenocarcinoma (Soda et al., 2007)

in particular, those in receptor tyrosine kinase (RTK) genes that drive oncogenesis (Thomas et al., 2007; Fabbro et al., 2012). Indeed, most of the kinase inhibitors at present on the market or in clinical trials target kinase receptors, such as EGFR, PDGFR, and HER2. About 150 kinase-targeted drugs are now in the clinical stage and many more are in preclinical development (Fabbro et al., 2012).

Although RTKs have been extensively investigated as primary targets in cancer therapeutics, the effector molecules downstream of them are also promising as targets in the next generation, since they or their signal pathways are frequently mutated in cancer. Among these, for example, Akt is a representative downstream mediator of various RTKs and its overexpression and/or activation has been observed in a wide variety of cancers (Altomare and Testa, 2005). Moreover, amplification of the AKT genes has been reported in lung, breast, ovarian and pancreatic carcinomas (Bellacosa et al., 1995; Kirkegaard et al., 2010; Dobashi et al., 2012). Therefore, Akt-specific inhibitors are in clinical or preclinical trials, although none has been marketed yet.

Thus, our current translational efforts have been based on these classic types of genetic analyses. Future translational efforts will be informed by the genome-wide, high-throughput technologies that have recently emerged.

3. Recent technological approaches to the identification of cancer targets

Cancers may be divided into two general types based on their molecular characteristics: (i) cancers having a specific genetic alteration and (ii) cancers having multiple complex karyotypic abnormalities without any specific pattern.

3.1. Cancers with a specific genetic alteration

3.1.1. Oncogene addiction

If a particular gene is critical to normal growth restraint, cancer will find a way to dysregulate it by any means, commonly by classic and "visible" genetic aberrations. Amplification, mutation and chromosomal rearrangements are predominant aberrations that have been detected by various scientific methodologies. During the process of oncogenesis, cancer cells become more dependent on the activity of that particular molecule, since other genes that physiologically play a similar role tend to become inactivated as cancer develops. As a result, cancer cells become "addicted" to a specific protein and/or a specific signaling pathway for their viability (Weinstein, 2002). This single aberrant gene is, in most cases, an oncogene or suppressor oncogene. Oncogenes can be activated via gene amplification or mutation, while suppressor oncogenes can be inactivated via deletion, point mutation, or epigenetic silencing by promoter methylation (Chin and Gray, 2008). Therefore, "visible genetic aberrations" have provided important clues for the clarification of cancer profiles. Furthermore, the detection of aberrant genes has been fruitful in identifying potential therapeutic targets. The landscape of dysregulation in the well-known oncogenic pathways has been unveiled by the technologies introduced below (Table 2), and the "tailored therapy" has become a reality.

3.1.2. Techniques for analyzing the cancer genome

Genomic analysis of various cancers is a current major effort in cancer research. In particular, characterization of DNA copy number, DNA sequence

Table 2. Techniques for analyzing the cancer genome.

Aberrations	Techniques	Chapters	References
DNA copy number	CGH	3.1.2.1	Kallioniemi et al., 1992
	FISH		Ooi et al., 2004; Dobashi et al., 2011b, 2012
	CISH		Simone et al., 2010
	FISH on TMA		Bayani and Squire, 2007
	CGH on TMA		Greshock et al., 2007
	MLPA		McMurray et al., 2008; Ooi et al., 2012; Homig-Holzel et al., 2012
Mutation	PCR/Sequencing	3.1.2.3	Chin and Gray, 2008; Schweiger et al., 2019
Translocation, Insertion, Deletion	FISH	3.1.2.2	Bayani et al., 2007
Methylation	methylation-sensitive PCR	3.1.2.4	Singer-Sam et al., 1990
	methylation-specific PCR		Herman et al., 1996
	Bisulfate sequencing		Eads et al., 2000
	Beads-chip method		Thirlwell et al., 2010
	Q-MSP		Qu et al., 2013

CGH, comparative genomic hybridization assay; FISH, Fluorescence *in situ* hybridization analysis; CISH, chromogen *in situ* hybridization analysis; TMA, tissue microarray; MLPA, Multiplex ligation-dependent probe amplification; PCR, polymerase chain reaction; Q-MSP, quantitative methylation-specific PCR.

and epigenomic modification are the focus of these efforts. Importantly, many of these analyses are possible using paraffin sections.

Formalin-fixed, paraffin-embedded (FFPE) tissues have both advantages and limitations in their use for molecular analysis. DNA tends to be fragmented into 100 to 300 base pairs due to the fixation process, however these lengths are sufficient for sequencing, *in situ* hybridization or methylation analysis when used in combination with polymerase chain reaction (PCR) amplification (Thirlwell et al., 2010). RNA is also degraded by fixation and the presence of RNases, but small scale gene expression arrays and microRNA analyses are still possible. Protein is also degraded by fixation and proteases, but these small fragments can be analyzed for proteomics or phosphoproteomics, as well as more basic quantification of expression by immunohistochemical staining. We will introduce the technical issues involved in each type of genetic and epigenetic analysis.

3.1.2.1. Copy-number aberrations. Analysis of gene or genome copy number alterations was initially made possible by comparative genomic hybridization assay (CGH), based on the technique of fluorescence *in situ* hybridization (FISH) (Kallioniemi et al., 1992). This analysis has particular relevance in cancer, where such aberrations are common. FISH was initially performed in cultured cells or fresh tissue using fluorescein-conjugated DNA probes. With the development of improved quantum efficiency and the stability of fluorescent and chromogen dyes, FISH and chromogen *in situ* hybridization (CISH) methods on paraffin sections were developed, which made it possible to define changes in gene number in individual cancer cells. For example, numerical changes in the *EGFR* gene in correlation with chromosome 7 were determined by dual-color FISH, utilizing a DNA probe specific for *EGFR* (specific for 7p12) conjugated with Spectrum-Orange™, and a chromosome 7-specific centromeric probe conjugated with SpectrumGreen™ that could be hybridized to paraffin sections (Ooi et al., 2004). In the CISH method, a digoxigenin-labeled *EGFR*-specific probe and a biotinylated chromosome-7-specific probe are used (Simone et al., 2010). These reagents are all commercially available. Thousands of bacterial artificial chromosome (BAC) clones are also commercially available and they can be used as FISH probes after incorporating fluorescence by nick-labeling (Dobashi et al., 2011b, 2012).

This technique is now performed on tissue microarrays, which allow the high-throughput screening of many cancers in one experiment and permits comparative analysis combined with histopathology (Bayani and Squire, 2007).

Recent new platforms for CGH are able to determine copy-number alterations in DNA sequences in microarrays and allow the quantitative evaluation of these changes (Greshock et al., 2007).

3.1.2.2. Structural aberrations. Structural DNA changes observed in cancer include deletions, insertions or translocations, and these have traditionally been identified by cytogenetic techniques, such as banding analysis (Nowell, 2007). It is now possible to analyze these changes on paraffin sections. Gross deletions and insertions are detectable using probes specific for the gene of interest, and manifested as loss or expansion of the gene signal. Translocations are identified using a dual-color FISH approach, whereby the genes involved in the translocation are differentially labeled and their fusion could be identified by co-localization of the probes. This is typically referred to as "fusion-FISH" (Bayani and Squire, 2007). Gene disruption is detected by yet another method. A FISH probe spanning the entire gene of interest or spanning the expected breakpoint, is hybridized to the specimen. The detection of a "split" signal indicates the break of the gene. Alternatively, a dual-color probe set for the 5'-side and the 3'-side of the breakpoint can be utilized for this "break-apart" analysis (Bayani and Squire, 2007).

3.1.2.3. DNA-sequence abnormalities (Mutations). Sequence analysis had traditionally been achieved using DNA extracted from fresh surgical material, and priming and incorporation of radiolabel by DNA polymerase (Chin and Gray, 2008). Since the 1990s, the spread of PCR and the development of non-radioisotopic methods have made this a relatively easy laboratory method. For example, determination of the *EGFR* genotype in NSCLC from FFPE tissues is crucial for the stratification of patients who might respond to TKI-therapy (Lynch et al., 2004). The *EGFR* genotype has been assessed by the successive rapid and efficient procedure "peptide nucleic acid-locked nucleic acid PCR clamp-based method", which targets specific hot spots in the *EGFR* gene conferring TKI-sensitivity or resistance (Lynch et al., 2004; Nagai et al., 2005). Additionally, the use of laser microdissection has allowed researchers to obtain FFPE tissue from cancers avoiding contamination by adjacent non-neoplastic tissue. This method has been developed since the 1980s, and can be combined with methods to extract DNA (Hood et al., 2005). These technological developments allow more specific analysis of cancer tissues from FFPE tissue than was previously possible.

The simultaneous genome-wide localization of copy number alterations and genomic mutations in FFPE tissues is also possible using second-generation sequencing techniques. These techniques have produced results from FFPE tissues comparable to those obtained with frozen samples, even with FFPE tissues preserved for a period of 18 years (Schweiger et al., 2009).

3.1.2.4. Epigenetic analysis. The term 'epigenetic' refers to the changes in gene expression and chromatin organization that are not the result of changes in DNA sequence (Herceg et al., 2013). Recent advances in cancer epigenetics have revealed several types of

chemical modifications, including DNA methylation and expression of microRNAs (Qu et al., 2013), which are major contributors to the oncogenesis and cancer progression (Jones, 2012; Bartke et al., 2013). Among them, aberrant DNA methylation in promoter regions is the most well-defined epigenetic hallmark of cancer (Sawyers, 2008; Goodell and Godley, 2013). DNA methylation analysis can be performed with biopsy or cytology specimens, thus it has been widely used in clinical applications.

DNA is predominantly methylated at cytosines in CpG dinucleotides, which are frequently clustered in GC-rich, 5' promoter regions in the genome termed "CpG islands (CGI)" (Qu et al., 2013). This covalent modification of DNA is associated with altered gene expression, in particular, aberrant DNA hypermethylation has been linked to inhibition of tumor-suppressor gene expression (Goodell and Godley, 2013).

Mapping of methylated regions in DNA initially relied on Southern hybridization approaches, based on the inability of methylation-sensitive restriction enzymes to cleave sequences that contain methylated site(s) (Herman et al., 1996; Eads et al., 2000). However, this method was limited by the incomplete mapping of restriction sites, ambiguous results caused by partial DNA digestion and the requirement of large amounts of high molecular weight DNA (Eads et al., 2000).

In more recent decades, many novel techniques have been developed to detect DNA methylation, including methylation-specific PCR, bisulfite sequencing (BS) and quantitative methylation-specific PCR (Q-MSP) (Qu et al., 2013). These second-generation techniques utilized the characteristic of sodium bisulfite treatment, which converts unmethylated cytosine to uracil, while leaving methylated cytosine unchanged (Frommer et al., 1992; Eads et al., 2000). Following bisulfite treatment, sequences including the particular loci of interest are subject to PCR amplification, and sequence differences resulting from the different DNA methylation patterns are identified by one of two different methods. In the first method, "methylation-specific PCR (MSP)" (Herman et al., 1996), methylated DNA is discriminated at the PCR step by the use of primers that anneal specifically with either the unconverted methylated or converted unmethylated sequence. In the second method, discrimination is made after the PCR reaction by the use of PCR primers that do not cover any CpG sites. This latter approach is exploited in all other bisulfite-based methods and results in the simultaneous amplification of all sequence variants arising from various patterns of methylation (Eads et al., 2000). "Bisulfite sequencing" methods generally refer to the determination of methylated sites by sequencing following bisulfite treatment.

Another useful bisulfate-based method is the combined use of methylation-sensitive enzymes and PCR. After DNA digestion with the restriction enzymes, PCR is performed with primers flanking the restriction site. Amplification occurs only if DNA cleavage is prevented by methylation (Singer-Sam et al., 1990).

Q-MSP is a highly sensitive assay, capable of detecting methylated alleles in the presence of a 10,000-fold excess of unmethylated alleles (Eads et al., 2000). The advantage of this method is that it allows the rapid screening of thousands of samples by PCR, without further electrophoresis. However, this technique requires expensive hybridization probes and the serial dilution of methylated and unmethylated controls to generate standard curves (Eads et al., 2000).

A more recent technique by "Illumina Infinium HumanMethylation27BeadChip" incorporates a ligation step of the template DNA to increase the molecular weight of the DNA, which is, therefore, applicable to genome-wide analysis using FFPE tissues (Thirlwell et al., 2010). The protocol includes, after the ligation step, bisulfite conversion, whole genome amplification, enzymatic digestion and hybridization to the array of beads on which oligonucleotide probes are conjugated (Thirlwell et al., 2010).

3.1.3. Routine characterization of gene aberrations in the pathology laboratory

When a cancer type is suggested as one that is dependent on a particular target, further validation should be carried out in order to translate this finding into the clinic. Histopathology has contributed to the characterization and the confirmation of pattern of DNA aberrations in cancer, and to the initial determination of therapeutic modalities. Since FFPE tissues can be preserved for decades, retrospective analyses of a vast archive of clinicopathologically characterized samples present in the pathology division of all large hospitals is possible.

3.1.3.1. Immunohistochemistry as the primary technology in the pathology laboratory. FFPE tissues have long been used for the confirmation of critical targets in oncology, almost exclusively by immunohistochemical analysis (IHC), since it has been the easiest and most reliable methodology for validation of the proteins. This strategy had a limitation, when sensitive and specific antibodies are not available. However, development of antibodies of higher quality has been achieved, and antigen recognition can be dramatically improved by heat treatment of paraffin sections, so-called "antigen retrieval" to unmask epitopes by reversing cross-links generated during fixation (Shi et al., 1991). However, IHC is still used primarily only with already well-characterized antibodies. Other obstacles include the inherent heterogeneity of cancer, variability in tissue collection methods (biopsy, surgery or autopsy), fixation methods, detection systems and criteria for evaluation. These obstacles may occasionally cause variability in IHC results. For example, upregulation of EGFR expression in NSCLC has been reported in the literature to range from 32 to 80%, depending on the report (Dobashi et al.,

2011a). In addition, a large retrospective study for IHC evaluation of estrogen receptor in breast cancer reported that inter-institutional observer variation produced a false-negative rate of 30–60% (Rhodes et al., 2000). Such variations are possible for any proteins, and thus IHC methods require thorough standardization for each target. Overall, given that IHC diagnostic tests are generally of low-cost, this approach is still suitable as a routine test.

One of the most successful examples of IHC informing the therapeutic approach to cancer is the case of rituximab therapy, a well-known, pathology-based molecularly-targeted therapy. Rituximab is a chimeric monoclonal antibody directed against the CD20 antigen on B-lymphocytes (Jazirehi et al., 2005). Thus, in addition to conventional histological subtyping of the lymphoma, analysis of CD20 expression by IHC and/or flow cytometry is directly relevant to selection of this therapy.

At present, IHC plays an important role in the clinic. For research purposes, tissue microarrays are an ideal platform for the rapid and high-throughput generation of an expression profile, enabling multiple samples and multiple targets to be comprehensively analyzed (Braunschweig et al., 2005).

3.1.3.2. IHC combined with FISH. Analysis of *HER2* gene aberrations previously required time-consuming genetic analyses, such as Southern blotting. Today, IHC are used and provide clear-cut criteria for the application of trastuzumab therapy in the clinic. Cancers that exhibit HER2 overexpression by IHC with/without additional detection of *HER2* amplification by FISH on paraffin sections are indicative of trastuzumab therapy (Dowsett et al., 2003). This is an excellent example of the potential of histopathology to contribute to molecularly-targeted therapy. In addition to breast carcinomas, in HER2-positive advanced cancers of the stomach, the protocol was established using IHC/FISH analyses on paraffin sections to select the patients eligible for trastuzumab therapy combined with cis-platinum or with capecitabine (Oshima and Masuda, 2011).

3.1.3.3. Use of FFPE tissues at multiple levels of cancer diagnoses. FFPE tissues are subjected to IHC, FISH, mutational as well as epigenetic analyses in both routine diagnostic tests as well as in basic research.

One example is the diagnosis and treatment decision for GIST. GIST is defined as a malignant spindle cell tumor harboring mutated *c-KIT or Platelet-derived growth factor receptor* (*PDGFR*). Diagnosis of GIST is ordinarily made by histological features together with analysis by IHC that shows positive staining for c-kit and/or CD34. When this diagnosis is made, treatment is either surgical resection or imatinib therapy. Imatinib therapy confers benefits in up to 80% of patients with advanced stage GIST, however its effectiveness wanes in half of these cases due to acquired resistance (Giuliani and Colucci, 2012). For cases that have acquired resistance, second-generation agents may be used, depending on the underlying mechanisms involved, such as amplification or acquisition of new mutations in *KIT*/*PDGFR*, or loss of c-KIT/PDGFR expression (Giuliani and Colucci, 2012). Amplification of *KIT*/*PDGFR* can be identified by FISH on paraffin sections using specific probes. Identification of secondary mutations can be determined using PCR amplification of DNA extracted from FFPE tissue, followed by sequencing. Loss of expression can be determined by IHC on paraffin sections. Therefore, FFPE tissues are used both at the initial diagnosis and in the analysis of resistance that may occur as a consequence of the primary therapy.

3.2. Cancers harboring multiple complex abnormalities

3.2.1. Complexity of cancer genes

Malignant transformation is a highly interactive process, involving multiple levels of genetic aberrations. Accordingly, individual tumors are often composed of diverse clones, subsets of which are driven by different oncogenic aberrations (Engelman et al., 2007; Alvarez-Calderon et al., 2013). In such cases, it may be hopeless to apply a single agent that effectively treats cancers driven by a single molecular driver. These cases require the more complicated task of identifying two or more targets and the corresponding therapeutic agents to which these cancers are sensitive. Even if several genes are found that show amplification, activating mutations or other aberrations in such cases, it will require much additional effort to validate the tumor dependence on these aberrant genes. It was shown in the initial clinical trials of Gefitinib, which involved a large number of NSCLC patients with EGFR overexpression, that only a small fraction of this population was responsive (Giaccone et al., 2004). Actually, except for those rare cases where the tumorigenic process is clearly driven by a single addicting gene, or a few candidate genes, identification of all relevant targets is almost impossible by the strategies introduced in the previous chapters. For this reason, a number of novel methods have been investigated to search a wider range of targets. One of these methods is “multiplex ligation-dependent probe amplification”.

3.2.2. Multiplex ligation-dependent probe amplification (MLPA)

MLPA is a high-resolution, multiple PCR-based method that can detect aberrations in copy number, DNA methylation and point mutations of up to 50 different genes in a single reaction (McMurray et al., 2008; Ooi et al., 2012). The data provide semi-comprehensive information that can be used to design a rational treatment strategy for cancers having heterogeneously amplified oncogenes (Fig. 1). For example, RTKs, including ERBB2, EGFR, FGFR and MET pathways

share downstream pathways, thus the blockade of a single pathway may be bypassed by activation of other pathways. This is seen in the amplification of *MET* in NSCLC as a mechanism of acquired resistance to TKIs (Engelman et al., 2007). MLPA probes recognize target sequences of 50 to 100 nucleotides, and therefore, small amounts of fragmented DNA from FFPE tissue are applicable. Moreover, MLPA can detect small areas of gene amplification when more than 25% of the cancer cells in a tumor sample harbor such amplification (Homig-Holzel and Savola, 2012). A commercial kit, the "Tumour-Gain kit" containing probes for 24 genes, along with other kits targeting various sets of genes, is available from MRC-Holland (Amsterdam, The Netherlands). The PCR products generated by MLPA are separated by a capillary sequencer (Homig-Holzel and Savola, 2012; McMurray et al., 2008; Ooi et al., 2012).

If more candidate genes need to be obtained to determine the pattern of gene expression in each case of cancer or in each type of cancer, trial for "seining" gene is preferable to comprehensively detect the candidates as gene expression signatures.

4. Gene expression signatures

The development of DNA-microarray technologies has enabled researchers to analyze the expression of tens of thousands of mRNAs in one experiment (Schena et al., 1995). Collecting such huge amounts of data has led to the generation of new analytical strategies. Systematic analysis of the gene expression patterns could allow us to visualize specific expression patterns or gene clusters that underlie specific cancer traits. This is the approach taken by the new technology of transcriptomics, from which we can derive gene-expression signatures, a new class of molecular diagnostic markers.

The most common procedure after having obtained a gene signature by genome-wide analysis, is to correlate patterns of gene expression with particular cancer traits (van't Veer and Bernards, 2008). Large-scale gene expression data sets can be analyzed by one of two methods. One is to find the subgroups (or clusters) of cancers with similar gene-expression patterns. Based on these similarities, cancer cases could be classified into subtypes that may share similar pathobiological profiles. This type of data analysis is called "hierarchical clustering" or "unsupervised classification" (Quackenbush, 2001). The second method is "supervised classification". Cases are divided into groups sharing the same clinical profiles or events (for example, with or without metastasis, drug sensitivity, etc.), and the genes that correlate with each group are explored. A current approach using FFPE tissues with well-characterized clinicopathological profiles is this latter type of analysis. Published examples include gene expression signatures that are associated with drug responsiveness or

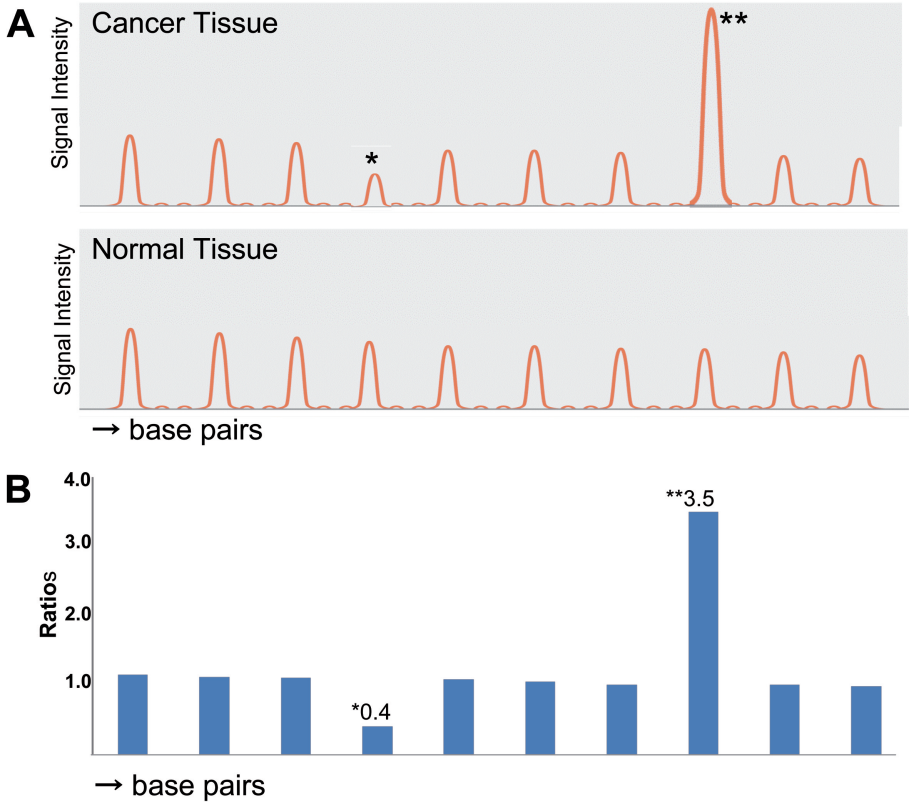

Fig. 1. A. A schematic presentation of the fragment analysis by multiplex ligation-dependent probe amplification (MLPA) method to detect copy number aberrations. The amplified products derived from different genes are separated and quantified by electrophoresis. The horizontal axis indicates the size of the PCR product generated from premixed primers in the Tumour-Gain Kit (MRC-Holland). The vertical axis indicates the amounts of the amplified products. The lower peak (*) suggests deletion of the gene or monosomy of chromosome in which the gene is located. The higher peak (**) suggests the amplification or polysomy. **B.** Data in A is expressed as relative ratios of amplified products from cancer tissues versus non-neoplastic tissues.

biological behavior in breast cancer (Perou et al., 2000; Sorlie et al., 2001; van't Veer and Bernards, 2008; Izzotti, 2012).

Alternatively, genes that may be relevant to a particular type of cancer can be selected from the literature. As with MLPA, sets of multiple 'suspected genes' are usually tested in these studies, and even small amounts of FFPE-derived RNA can be analyzed by RT-PCR. Inevitably, the outcome is not more than the present state of knowledge (van't Veer and Bernards, 2008).

Although recent large-scale DNA-sequence analyses have identified thousands of candidate genes that may play critical roles in human cancers, little of this data has been translated into clinical practice (Sawyers, 2008). Achieving the next step by rapid application of genetic information to new diagnostic tests requires validating the dependence of a cancer on a particular target gene, clarifying its prognostic utility and demonstrating the sensitivity to a drug directed against the target.

In the next chapter, we will introduce several strategies utilized by the other "-omics", and the role played by FFPE tissues.

5. Other "-omics" and Histopathology

Transcriptomics, i.e., gene expression signatures, generates a huge amount of data and has provided a powerful tool for drug target identification. Nonetheless, as proteins are the primary effectors of cell physiology, protein-based assays are expected to be more clinically relevant (Brennan et al., 2010; O'Leary et al., 2013). Hence, our ultimate goal is to elucidate the functions of the products of the expressed genes identified by transcriptomics. This means the clarification of each protein with regards to its quantification, localization in the cell, determination of protein–protein interactions and elucidation of post-translational modifications, such as glycosylation and phosphorylation. This goal is the basis for the development of the other "-omic" technologies, such as proteomics and phospho-proteomics.

5.1. Proteomics

Since the total gene transcription signature derived from transcriptomics does not fully capture the profiles of a particular cancer or subset of cancers, technologies that analyze protein expression profiles have recently been of great interest. Combining established two-dimensional electrophoresis with subsequent mass spectrometry (MS) analysis, together with the ability to systematically produce specific antibodies, has provided new tools for the high-throughput functional analysis of cell protein expression (Brennan et al., 2010). In addition to this strategy, IHC has become a more powerful tool in the clinical laboratory due to its easy availability, low cost and refined applicability to FFPE tissue. IHC can be used for the systematic investigation of the human proteome in a wide range of high-throughput assays, collectively known as IHC on tissue microarrays (Brennan et al., 2010). These technologies allow the translation of protein targets identified in gene expression studies into clinically applicable IHC-based panels for cancer (Nielsen et al., 2004). Moreover, these technologies could be enhanced by further development of proteomics and phosphoproteomics, realization of which is just around the corner.

5.2. Phosphoproteomics

5.2.1 Phosphoproteomics at the "bench"

New tools in proteomics are being developed to reveal the complex networks of dynamic and interconnected signaling that occurs in cells. Many cellular processes are regulated by signal transduction, such as those involving the reversible phosphorylation of proteins. Aberrant phosphorylation of serine, threonine and tyrosine residues has been implicated in cancer and other disorders (Pawson and Nash, 2003; Tedford et al., 2009). Phosphoproteomics basically deals with the detection and quantification of phosphoproteins and phosphopeptides, and determination of phosphorylation sites. The most prevalent methodology is to generate phosphopeptides by tryptic protein digestion followed by MS (Tichy et al., 2011). Although this technology can be hindered by the relatively low amounts of phospho-proteins present in extracts, this obstacle can be overcome by techniques for their preferential enrichment (Harsha and Pandey, 2010). One early enrichment method involved the use of antibodies directed against phosphotyrosine (PY) residues, successively followed by the application of anti-phosphoserine (PS) and phosphothreonine (PT) antibodies (Tedford et al., 2009; Harsha and Pandey, 2010). Recently, new advances in phosphoenrichment have been made, e.g., using immobilized metal affinity chromatography to exploit the high affinity of phosphoserine for ferric ions and gallium (Posewitz and Tempst, 1999; Stensballe et al., 2001; Harsha and Pandey, 2010).

Other approaches include the global identification of kinase substrates via peptide microarrays or protein microarrays (Harsha and Pandey, 2010). This methodology can be utilized in cancer phosphoproteomic studies to identify hyperactivated kinases (Leitner et al., 2011; Tichy et al., 2011).

The quantitative comparison of phosphoproteins in cancers can be used to evaluate the therapeutic effects of an agent or to profile signaling cascades unique to the particular disease phenotype (Tedford et al., 2009; Derouiche et al., 2012).

5.2.2. Phosphoprotein-specific antibodies

In the past decade, a critical reagent enabling phosphoproteomics has been antibodies specifically directed against the phosphorylated forms of proteins.

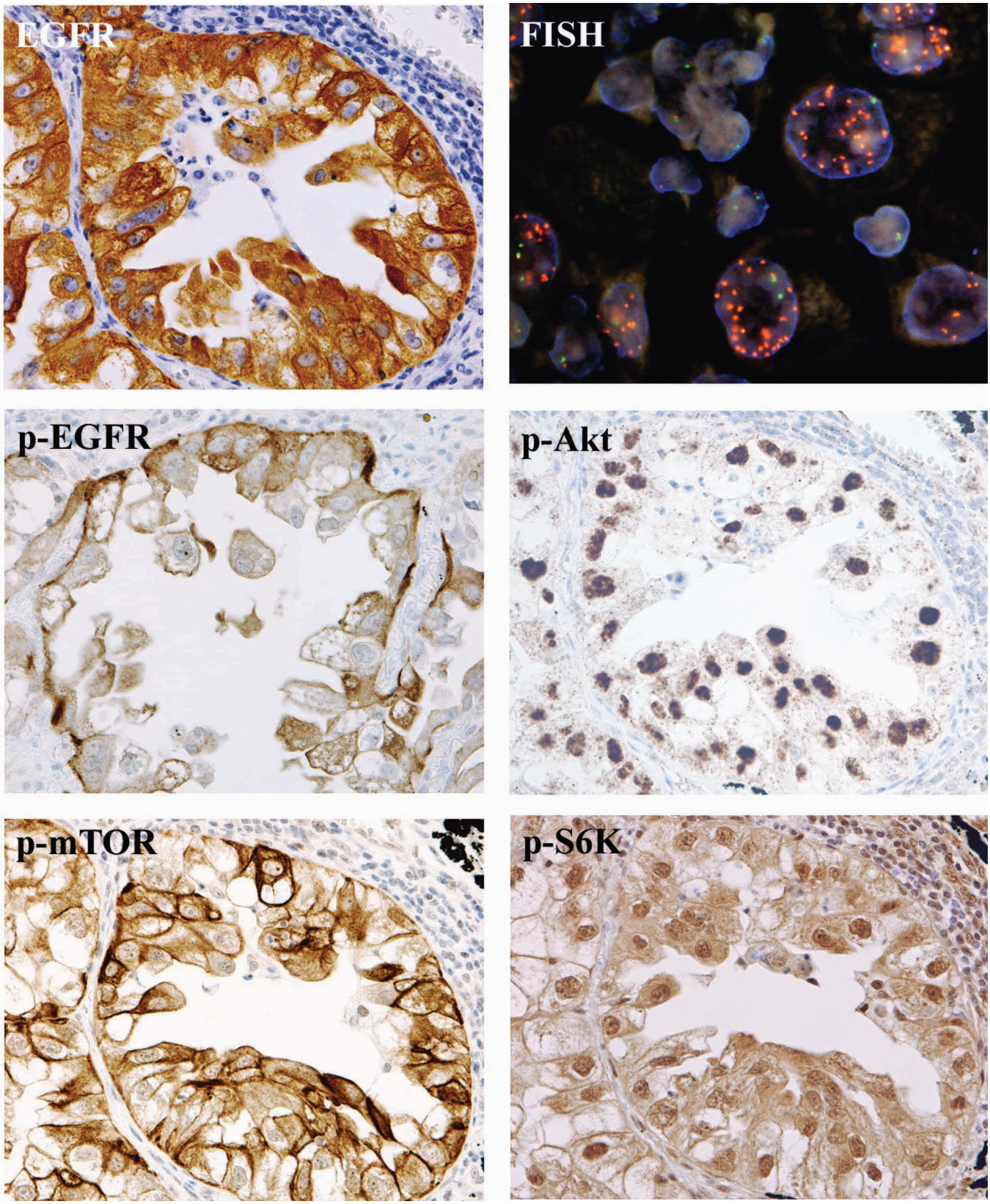

Fig. 2. Immunohistochemical staining for EGFR and activated proteins in the EGFR/Akt/mTOR pathway. Staining was performed using anti-EGFR and anti-phospho-specific antibodies. A case of adenocarcinoma of the lung (harboring mutation of L858R) showed positive staining for EGFR, and activation of EGFR and downstream proteins. Fluorescence *in situ* hybridization analysis (top right) revealed scattered-type, high-level amplification of the EGFR gene. Abbreviations: p-EGFR, phosphorylated EGFR; p-Akt, phosphorylated Akt; mTOR, mammalian target of rapamycin; p-S6K, phosphorylated p70S6 kinase. Reproduced from "Paradigm of kinase-driven pathway downstream of epidermal growth factor receptor/Akt in human lung carcinomas" by Y. Dobashi, et al., Human Pathology 42, 214–226, 2011. Copyright Elsevier Limited. Reproduced and modified with permission. x 200

These antibodies are raised against epitopes consisting of PS, PT and PY residues together with adjacent amino acid sequences. A large body of these have been developed as monoclonal antibodies in rabbit and are commercially available. These antibodies can be used not only for IHC (Fig. 2), but can be used for direct phosphoprotein blotting, replacing the more classic method of immunoprecipitation by specific antibody followed by immunoblotting with anti-PS, anti-PT or anti-PY antibodies (Dobashi et al., 2011b, 2012).

5.3. Proteomic analysis on FFPE tissues

Proteomic investigations using FFPE tissues have been hampered by the high degree of covalent cross-linking caused by formalin fixation, predominantly affecting lysine side chains (Ostasiewicz et al., 2010). Therefore, it has long been believed that proteins in FFPE tissues may not be suitable for MS-based proteomics. However, the past few years have witnessed the characterization of a large body of proteins and

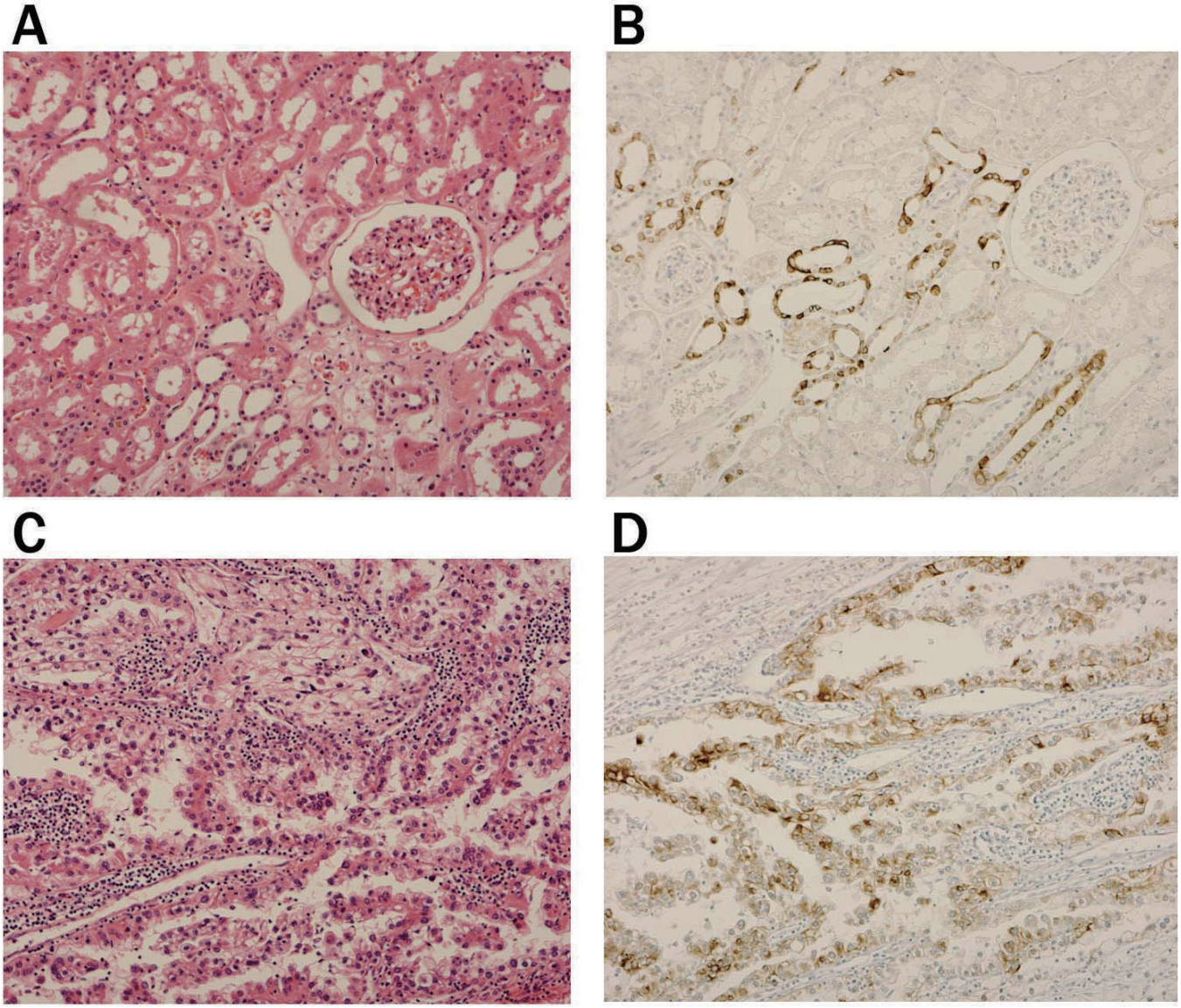

Fig. 3. Localization of phosphorylated forms of mammalian target of rapamycin (p-mTOR) in renal cell carcinoma. **A, B.** Histological features of renal cortex adjacent to the cancer nodule (**A**. hematoxylin-eosin stain) and intense staining in renal tubules by immunohistochemical staining for p-mTOR **(B). C, D.** A portion of renal cell carcinoma, showing papillary structure revealed activation of mTOR (**C**. hematoxylin-eosin stain, **D**. p-mTOR stain). x 100

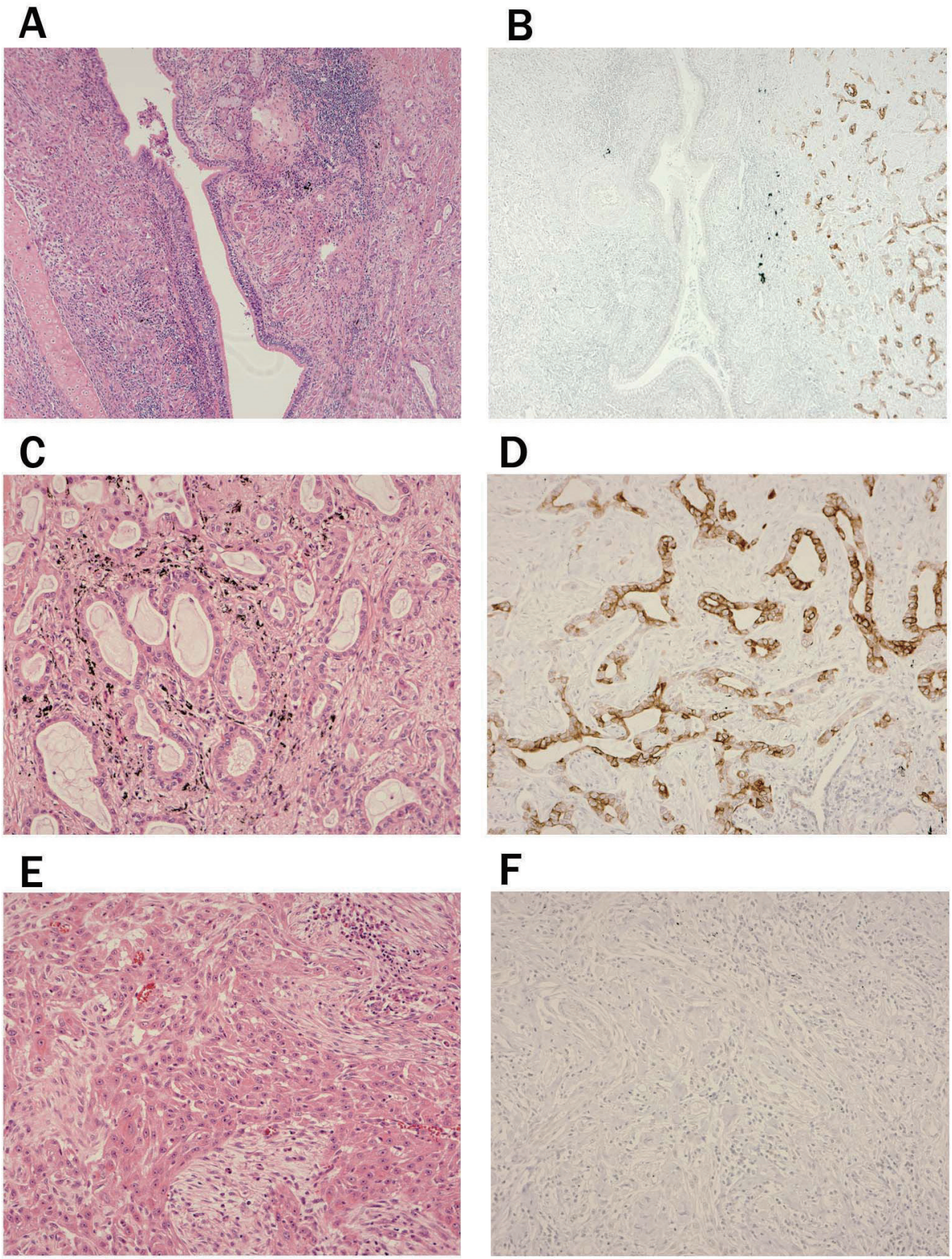

Fig. 4. Localization of phosphorylated form of mammalian target of rapamycin (p-mTOR) in adenosquamous carcinoma of the lung. **A.** Histological features of adenosquamous carcinoma of the lung shown by hematoxylin-eosin staining. **B.** Intense staining in adenocarcinoma (right half), but not in squamous cell carcinoma (left half) by immunohistochemical staining for p-mTOR. **C, D.** Component of adenocarcinoma reveals activation of mTOR. (**C**. hematoxylin-eosin stain, **D**. p-mTOR stain) **E, F** Squamous cell carcinoma indicates absence mTOR activation (**E**. hematoxylin-eosin stain, **F**. p-mTOR stain). A, B, x 40; C-F, x 100

phosphoproteins in FFPE tissues. Furthermore, hematoxylin-eosin staining turns out to have little effect on the quality of the preserved proteins, which allows the proteomic analysis of histopathologically confirmed tissues (Ostasiewicz et al., 2010).

One protocol termed "the filter-aided sample preparation method" allows the identification of 5000 proteins from one sample in a one day experiment (Wisniewski et al., 2009). Moreover, this procedure may enable the quantitative mapping of phosphorylation sites. Thus, IHC using specific anti-phosphoprotein antibodies, in combination with MS-based techniques, constitutes a novel, highly complementary strategy for cancer target identification (Brennan et al., 2010).

6. Irrelevance of "bench" and "bed"

Since cancer is a heterogeneous disease that encompasses a wide variety of cell types, effective therapeutic approaches require the categorization into particular subtypes: The heterogeneity of cancer presents certain challenges. In a study for drug sensitivity, for example, we may find that one sample group consists of a mixture of several subtypes of cancers presenting contrasting phenotypes, e.g., different histopathological types, and may be a mixture of a major subtype showing higher sensitivity for the agent plus a minor subtype having poor sensitivity. A common problem is that such studies often do not provide detailed histopathological classifications in the report. An example of this problem may be seen in the case of the Rapalogs, derivatives of the Ser/Thr-kinase inhibitor (STKI) rapamycin. Rapalogs include temsirolimus, everolimus, ridaforolimus and other analogs that block the activity of mammalian target of rapamycin (mTOR), a major downstream mediator in the growth factor receptors/PI3-K/Akt pathway. Rapalogs have been approved for the treatment of renal cell carcinoma and pancreatic neuroendocrine carcinomas (Dobashi et al., 2011c; Yao et al., 2011). Indeed, intense staining of the phosphorylated form of mTOR (p-mTOR) could be observed in renal tubules by IHC (Fig. 3A,B). However, while the p-mTOR signal could be observed in a subset of those two kinds of carcinomas, it was not always seen throughout the entire nodule of the carcinoma (Fig. 3C,D). Furthermore, cases positive for p-mTOR do not necessarily account for a major proportion of total cases of those carcinomas, with frequencies of less than 50% of total cases (Kruck et al., 2010; Han et al., 2013). In this case, the evidence provided from IHC analysis on paraffin sections would have suggested that the drug would "not be effective". In contrast however, the results of the clinical trials were reported to be "promising".

In yet another case, a large study on NSCLC reported disappointing results with rapalogs: concurrent administration of EGFR/mTOR inhibitors (gefitinib/everolimus) in a Phase II study produced only a partial response rate of 13%, which did not pass the predetermined response threshold required for further study (Price et al., 2010). The pitfall of this study is that the samples contained a mixture of adenocarcinoma, squamous cell carcinoma and large cell carcinoma, and each histopathological type was not examined individually. Several other studies of NSCLCs have shown intense IHC staining for p-mTOR ranging from 51 to 74% of the total cases (Gridelli et al., 2008; Pal et al., 2008; Dobashi et al., 2009, 2011a,b). However, the activation patterns of mTOR in NSCLC vary depending on the histological type (Fig. 4). While mTOR was activated approximately in 90% of the cases of adenocarcinoma (Fig. 4C,D), the incidence was 40% in squamous cell carcinoma (Fig. 4E,F) and much less in small cell carcinoma (10.0%) (Dobashi et al., 2009, 2011a,b). Despite the observation from IHC analysis that mTOR signaling is upregulated in adenocarcinoma, suggesting its possible critical involvement, the rapalog was not approved as it was judged "not so promising for NSCLC". This conclusion may be different if such a large study is more carefully performed based on the histological classification and the results of pathological analysis. Thus, there may be room for improvement in how to design and evaluate such clinical studies, by combining with IHC analysis of patient' samples.

7. Concluding remarks

The critical current issue underlying the development of more effective cancer therapies is a better understanding of the molecular mechanisms driving oncogenesis and clarifying the responsiveness of different cancer phenotypes to potential therapeutic agents. Characterization of cancers using genome-wide screening is advancing, as are efforts to integrate these findings with information about the responsiveness of these cancers to different therapeutic agents. Together, these efforts are paving the way for novel therapeutics. Integrating histopathological information is an important component of this effort, and ideally the molecular analyses should be undertaken in the pathology laboratory. FFPE tissue is a powerful tool to characterize previously known aberrations, both by conventional and newer methods; IHC, tissue microarrays, PCR-sequencing, FISH/CISH and MLPA. The advantages to these approaches are the relatively low cost and the relatively short time required. Furthermore, the ability to make use of almost any available FFPE tissues in this methodology is a particular advantage, since cancer cells can be preserved as long as the tissue remains in the paraffin block. The biggest challenge facing pathologists today is how to integrate the information obtained from the molecular analyses into the pathology-based tests. Our understanding of the utility of FFPE tissue is only now beginning to emerge.

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