# MOLECULAR PATHOLOGY IN DRUG DISCOVERY AND DEVELOPMENT

Edited by
J. Suso Platero

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*To Begoña, Pablo, Santiago, Maria, Laura, Lucas, and Cecilia the reason for my existence and to Joel C. Eissenberg, who taught me how to do science*

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# PREFACE

During the last few years there has been a great deal of public interest in the area of personalized medicine. News articles, scientific magazines, and entire books have been dedicated to the subject. While a lot has been said about the subject, there is little done in practice. Nowadays there are only a few examples of personalized medicine. One of them is the use of the HER2 diagnostic test, in breast cancer patients, in order to treat them with Herceptin, a drug that works well in that subpopulation. Other tests, like the estrogen receptor (ER) or the progesterone receptor (PR), are also used to put breast cancer patients in hormonal therapy. All these diagnostic tests could be characterized as molecular pathology tests.

My intent in putting this book together was to show others how one can develop new molecular pathology tests for use in personalized medicine. I have used the process of drug discovery and development as the outline of the book for a simple reason, the discovery of the molecular pathology test could be done at the same time that the drug is been discovered. In Chapter 1, Dr. Franz Fogt gives an overview and historical perspective of the field of molecular pathology, and I follow it with a simplified overview of the drug discovery and development process. Chapter 2 follows with a view of the drug discovery process and how molecular pathology could be used to identify and validate new drug candidates. Chapter 3 introduces the reader to the world of biomarkers, and how biomarkers could be found using transcriptional profiling. These biomarkers can then be used as surrogate endpoints, and molecular pathology could play a significant role in validating these biomarkers and developing tests for use in hospitals. This chapter is followed by examples of molecular pathology in safety assessment in the area of toxicology. It also gives an overview of toxicology and its methods to identify off-target liabilities of drugs in both small molecules and biological compounds. Chapter 5 looks at toxicogenomics, a new way of doing toxicology by looking at transcriptional profiling to identify genes that are relevant to the safety of compounds. This chapter is followed by the use of molecular pathology in clinical trials. Examples of how molecular pathology assays have helped identify the right dose for different drugs are shown. Not only is molecular pathology useful in finding the right dose but also in finding the right patients for treatment, which is discussed in Chapter 7. Here again is the area of personalized medicine that is directly affected by molecular pathology. Several examples are shown of how this is done today in the clinic. The following chapters deal more with direct

applications of molecular pathology. Chapter 8 shows several examples of usage of molecular pathology in molecular therapy. Chapter 9 is a practical approach on how to do immunohistochemistry (IHC), one of the most important and useful techniques in molecular pathology. This chapter also indicates if you do not have the expertise in house how to use other companies, contract research organizations, to do this type of work. The last two chapters look more at the future of molecular pathology. Chapter 10 deals with the quantification of the colorimetric signal while Chapter 11 looks at fluorescence as a way to quantify and normalize the signal.

Color representations of selected figures in the book are available as pdf files at the following ftp site address:

ftp://ftp.wiley.com/public/sci_tech_med/molecular_pathology

I want to thank all the authors for their work. Each chapter has the contributions of people who are truly experts in their fields. Also, thanks to Jonathan Rose at John Wiley & Sons for his patience in guiding me through the whole process.

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# 1

# MOLECULAR PATHOLOGY AND DRUG DEVELOPMENT

Franz Fogt and J. Suso Platero

## 1.1. GENERAL PATHOLOGY

The histopathologic assessment of tissues and, for that matter, body fluids serves to diagnose alterations and disease state and helps to categorize and collect information about disease. The pathologic assessment of tissues and organs itself is a stepwise process of progressive analysis of the present disease, and the next possible finding one can describe with (relative) certainty. This is, naturally, only possible when a sufficient amount of tissue is submitted to pathology. If fluid material, only cells present in that specimen can be assessed and further evaluation can mostly not be done with certainty. For the diagnosis of a colon carcinoma, a microscope is rarely necessary. When opened, the colon will reveal the tumor, the size, and, at least semiquantitatively, the invasive depth. However, to assess the correct depth and the type of carcinoma, a section of the tissue must be reviewed with the microscope. The next necessary diagnostic step to categorize, grade, and stage the lesion is the review of the lymph nodes as to their involvement by metastatic disease. Traditional histopathology uses the morphologic aspects of tissue and cellular arrangement to provide diagnosis as to the cellular origin of malignant tumors.

Similarly, morphologic features can be used to predict behavior and outcome of malignant tumors and can influence the way certain tumors are treated. This

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is illustrated by the relative bland morphology of bronchioloalveolar carcinomas of the lung with a relatively benign outcome compared to the guarded outcome of poorly differentiated small-cell carcinomas of the same organ. In the case of colorectal carcinomas, the morphologic aspect of tumor transgressing through all layers of the bowel wall and its presence as metastatic tumor within lymph nodes indicates a higher stage of disease and predicts a guarded outcome. At the same time, based on such information, specific treatment, that is, chemotherapy, radiation, and surgery can be initiated.

## 1.2. GENERAL ASPECTS

Molecular pathology generally describes the aspect of pathology that is removed from the purely histologic aspect of diagnosis and uses information on the molecular level for diagnosis and prediction of outcome. Thus, the molecular aspect of pathology deals with identification of genes and the subsequent change in cellular architecture and expression of proteins in a given disease. Taken in such broad terms, molecular pathology is something pathologists have done for a long time, even before biochemical techniques were invented to analyze cellular DNA. Application of molecular pathology was used to imply the analysis of cellular structures at the electron microscope level or the analysis of proteins within the cell (Roizin, 1964). Staying with malignant tumors, identification of specific proteins within tumor cells can aid in the diagnosis of cellular origin, which may be important for both diagnostic and therapeutic purposes. The presence of tumor within the lung that expresses prostate-specific antigen (PSA) will undoubtedly define this tumor as a metastatic lesion and exclude a pulmonary primary tumor. These proteins may be visualized both by immunohistochemistry, that is, staining with immunohistochemical stains for PSA in case of prostate carcinoma, or by histochemical methods, that is, visualization of mucins with mucicarmin stain for other lesions. These examples use the expression of normal proteins in a tumor, which is, naturally, gene driven. Further assessment of tumors can identify expression of proteins that are not normally expressed in normal cells and, again, be of diagnostic use. The wild type of the p53 protein is a short-lived protein. The probability to have wild-type protein present in a given cell at a given time is quite low, and, thus, staining of tissues that contain wild-type p53 will result in a negative staining. Mutated p53, on the other hand, has a long half-life, will be present in tissues containing that protein, and will stain positive for p53, indicating its abnormal presence (Rom et al., 2000).

## 1.3. MOLECULAR PATHOLOGY, THE MOLECULAR WAY

The genetic code represents a specific code of four desoxynucleotides, which combine with complementary strands of DNA. When isolated from the nucleus, DNA usually breaks easily at random areas, resulting in DNA strands of various

lengths making it difficult to impossible to analyze a specific area of a DNA strand. The invention of restriction endonucleases in the 1970s allowed for the first time to produce specific DNA fragments of defined areas and length. This allowed identification of specific DNA sequences when those DNA strands were, after detachment from their complemetary strands, hybridized with marked deoxynucleotide oligonuceotide complementary strands. Such strands with known sequence, usually radioactive marking, have been produced in the laboratory and could be performed with the enzyme DNA polymerase. The idea that DNA polymerase was also able to multiply in an exponential fashion a specific strand of DNA flanked by oligonucleotide primers was first introduced by Mullis in the early 1980s (Mullis, 1990). The first methods to use polymerase chain reaction (PCR) for diagnostic purposes were applied to the diagnosis of hemoglobin diseases in the mid-1980s (Saiki et al., 1985). This technique allows one to analyze the genetic abnormalities that make up specific parts of the DNA and to analyze alterations that are represented within the genome. One such technique would be the analysis of loss of heterozygosity (LOH). Here, one assumes that all genetic alleles are present in duplicate within a given somatic cell. Those alleles may be identical, called homozygous, or slightly different, called heterozygous. In tissues with heterozygous alleles comparisons can be made to malignant tissues within the same body by testing for LOH. Primers flanking the alleles of choice are produced and the DNA strand of interest is multiplied and analyzed. If the normal tissue continues to show two distinct allele bands but the tumor has lost one of the two bands, the tumor is said to show LOH of the given gene tested. Other abnormalities may be analyzed with the same basic technique by sequencing the DNA strand of interest to assess whether the base pair sequence differs from the normal tissue.

### 1.3.1. Loss of Gene Expression

The previous example of p53 expression representing the assessment of cellular proteins is the result of genetic alterations, specifically the loss of genetic function of the *p53* gene. Typical examples for loss of function of genes are mutation, methylation, loss of heterozygosity, and so forth. Inappropriate silencing of genes through the mechanism of methylation has been described specifically in tumors of the gastrointestinal tract in patients with hereditary nonpolyposis colorectal cancers (HNPCC) (Chen et al., 2007). Methylation of genes or promoter regions of genes can inhibit the function of the genes or promoter regions and result in loss of genetic function without change in the sequence of the genetic code. In vertebrates a methyl group is bound to the 5-carbon of a cytosine base, which is located next to a guanine through the enzyme DNA methyltransferase, resulting in a CpG dinucleotide pair. The effect of methylated DNA on transcription is probably not related to its physical change but more by abnormal protein binding capacities (Wajed et al., 2001). Microsatellite instability (MSI), on the other hand, is a situation in which a microsatellite allele changed the number of repeat units resulting in a change of length. If this change is present in a given neoplasm, it can be concluded that this neoplasm is monoclonal. One of the first lesions in

which MSI has been observed was HNPCC, where it was associated with defects in the DNA repair mechanism. Involved genes of MSI were *hMLH1*, *hMSH2*, and *hMSH6*. Normal DNA repair will correct the mutation, but in patients with deficient mismatch repair function, a high percentage of mutation risk is present, which can also affect coding of DNA regions. If regions involving proliferation or programmed cell death are affected, cancer can develop. This genomic expression of MSI can be tested in family members, and affected persons can undergo regular surveillance examinations (Baudhuin et al., 2005). Such testing involves the analysis of tumor and nontumor tissue and the comparison of dinucleotide repeats between the tissues. To evaluate for low microsatellite instable and high microsatellite instable patterns, five markers have to be tested. If at least two markers show mutational pattern, the tumor is said to be high microsatellite instabile (Fig. 1.1).

Other tumors may show loss of heterozygosity of specific tumor suppressor genes. Here, two alleles of the tumor suppressor gene are present with one of the alleles being genetically altered, that is, silenced. If the second allele, which must have been genetically stable, is damaged by a second hit, that allele may no longer be present but only the one previously nonfunctional. Those changes may be present as germ cell mutations, such as hereditary retinoblastoma or acquired as in cases of usual colorectal adenomatous polyps.

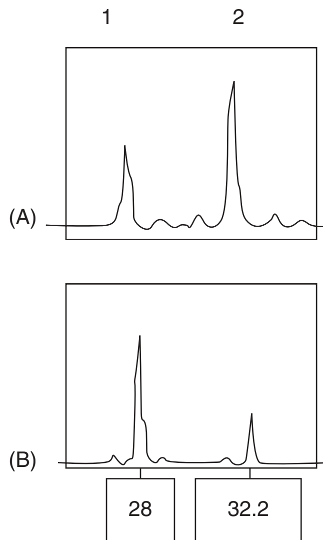


Figure 1.1. Microsatellite instability. Set (A) represents normal tissue. Lanes 1 and 2 show almost equal size of the two heterozygous alleles 28 and 32.2, respectively. Set (B), representing tumor, shows a >50% loss of height at marker 32.2 (lane 2). This loss represents LOH. (Courtesy of Zoran Budimlija, Department of Forensic Biology, Office of the Chief Medical Examiner, New York, New York.)

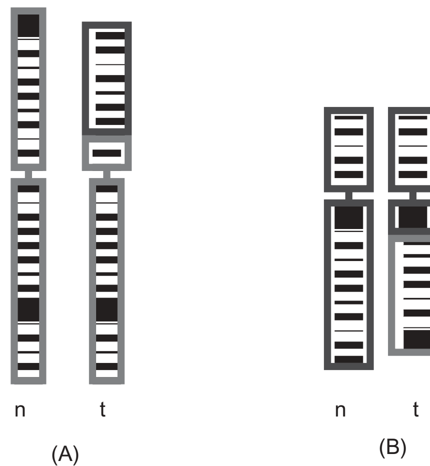


Figure 1.2. Translocation. Translocation defines the exchange of fragments of DNA between chromosomes. The figure shows normal chromosomes (A) and (B) (n) and the same chromosomes after translocation of fragments of DNA has been taking place (t).

### 1.3.2. Translocations

Molecular pathologic analysis of specific tumors is also of diagnostic use both in routine cases, such as malignant lymphomas, and in the diagnosis of soft tissue sarcomas, which may show signature genetic aberrations diagnostic for a specific disease entity and possibly predictive for specific tumor behavior. In lymphomas, genetic analysis may reveal specific translocations and predict the development of genetic alterations that make mucosa-associated lymphoid tissue (MALT) tumor of the stomach no longer susceptible for antibiotic therapy. Translocation is the rearrangement of fragments of chromosomes that are nonhomologous. They are usually denoted as t(A;B) indicating that the translocation is between chromosome A and B and ($q_x$; $p_x$), indicating the location on gene *A* and *B*, respectively; p and q stand for short and long arm of chromosomes (Fig. 1.2).

In soft tissue sarcomas, histologic similarity between spindle cell sarcomas may make it difficult to diagnose with certainty the presence of synovial sarcoma. The tumor-specific translocation (tX;18) can aid in the diagnosis of synovial sarcoma and lead to specific therapeutic intervention (Fig. 1.3).

### 1.3.3. Detection of Pathogens

In nonmalignant aspects of disease, molecular pathologic techniques may be able to detect specific pathogens, that is, viruses and bacteria, associated with disease. The typical examples here are the genetic detection of specific viruses in patients with human papilloma virus infections. Here, the routine genetic testing for specific virus types has not only led to therapy and surveillance for specific patient

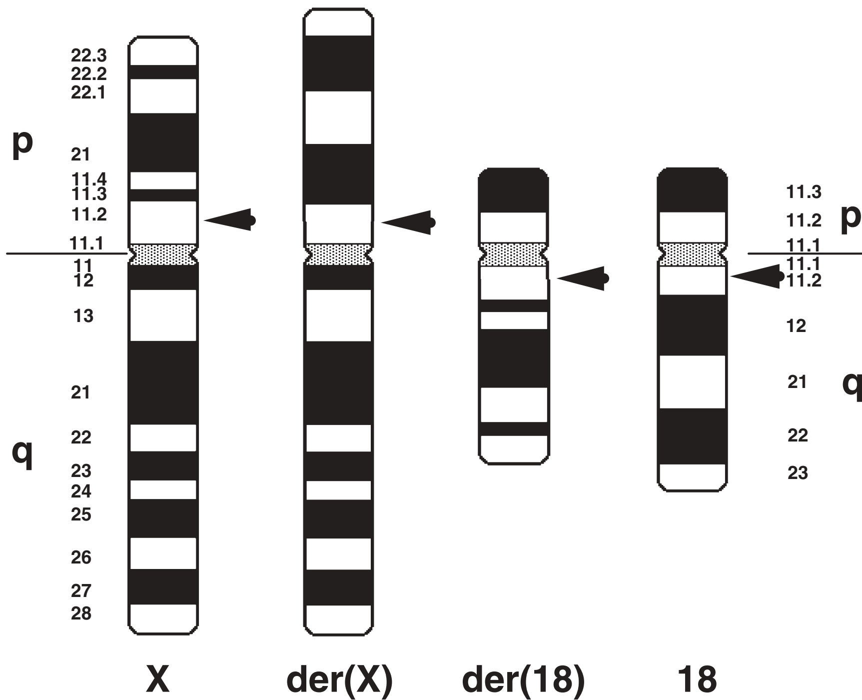


Figure 1.3. Synovial sarcoma x;18 Translocation. t(X;18)(p11.2;q11.2) in synovial sarcoma G-banding; substitution of the 8 last amino acids of SYT by 78 amino acids of SSX1 and SSx2. (Courtesy of Dr. Ferederic Barr, University of Pennsylvania, Philadelphia.)

populations, but is, at least indirectly, involved in the recent successful development of vaccine for high-risk human papilloma virus infection. In addition, the molecular biologic techniques can be used to compare genetic information of current pathogens with historic pathogens, such as comparison of the current bird flu H5N1 and the virus that caused the 1918–1920 influenza pandemic (Wang et al., 2007).

### 1.3.4. Forensic Identification

At the same time, molecular pathologic techniques may be applied to assess for presence of specific normal genes when attempting to identify origin of tissues, that is, identification of unknown persons/victims. The theory is that, although the DNA between one human to the next is rather identical, short repetitive DNA areas exist, which are known as polymorphisms. Short tandem repeats (STR) are such short sequences of DNA, 2 to 5 base pairs in length, which are quite individual in different persons. By testing 9 to 15 such areas of STR, the probability that one person shares the exact STR with another person within a specific

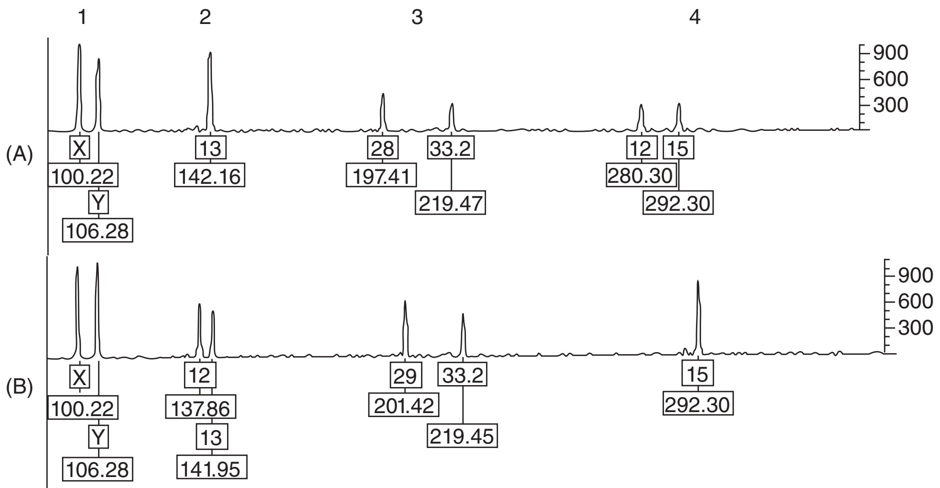


Figure 1.4. Forensic STR testing. Two runs of short tandem repeats. Individual A and B. STD 1 and 3 are shared by both individuals. For STD 2, individual A is homozygote (single peak at point 13), whereas individual B is heterozygote with two peaks at 12 and 13. Similarly at STD 4, individual A is heterozygote with two peaks and individual A homozygote with one peak. These features indicate probability that the individuals tested are not identical.

regional pool of persons decreases at least logarithmically and allows the determination of the origin of specific tissue samples (Fig. 1.4) (Tamaki et al., 1996).

### 1.3.5. Protein Changes

Assessment of specific DNA alterations, such as mutations, may indicate a specific tumorigenic pathway, but the changes in the DNA may not necessarily predict whether the altered DNA leads to altered protein expression downstream. Areas in which genetic alterations and specific protein expression patterns have been observed are specifically evident in the expression of c-Herb in carcinomas of the breast or in staining for *hMLH1* in colorectal carcinomas of patients with familial nonpolyposis colorectal cancer. Both have been used to specify further diagnosis of disease but also for therapeutic application. In patients with proven c-Herb expression, specific treatment options can be used, which are not applicable in tumors not expressing this protein. In patients with familial colorectal cancers that are microsatellite instable, the diagnosis of MSI will lead to more frequent surveillance and examination of other organ systems prone to develop tumors in this state of disease. Testing for epidermal growth factor receptor (EGFR) in patients with epithelial carcinomas has led to treatment options with antibodies to EGFR in expression-positive patient populations.

Testing for the presence of altered, that is, increased or mutated, messenger ribonucleic acid (RNA) may provide more insight into the effect of genetic

DNA-based alterations on the development of tumors. Messenger-RNA-dependent protein expression may again provide additional insight into the alterations that resulted from the original DNA damage. Thus, the circle to the analysis of cellular proteins is closed.

### 1.3.6. Other Methods of Detection

Beside the immunohistochemical analysis of cellular proteins, newer techniques allow for the analysis of vast amounts of proteins present in a given tissue substrate. Proteomic analysis uses two-dimensional gel electrophoresis to dissociate proteins that can subsequently be isolated and sequenced both for identification of the native protein and the presence of mutated forms of a specific protein. Newer proteomic techniques allow for dissociation and analysis with a one-step procedure. Protein signatures can be used to identify tumors or predict presence of tumors when found in surveillance specimens, for example, serum analysis. Historically, the time from the determination of the correct number of human chromosones in 1956 (Tjio and Levan, 1956) to the discovery of constant genetic alterations in malignant disease process such as chronic myelogenous leukemia (Nowell and Hungerford, 1960) was very short. To date most human genes have been deciphered and a vast number of disease processes has been linked to specific genetic alterations, and a much fewer number of those genetic alterations have been specifically used for pharmacologic application.

## 1.4. APPLICATION OF MOLECULAR PATHOLOGY

How molecular pathology has aided in the understanding of the development of the malignant phenotype may be underscored by the discovery of differences in colorectal carcinomas. It has been shown previously that colorectal carcinomas develop frequently from adenomas, which are adenomatous polyps found in the colon of patients. At the same time, there is a familial disease in which patients develop thousands of adenomas and have a 100% certainty to develop invasive tumor by age 50. Early molecular pathologic studies have shown that these tumors follow a specific stepwise progression with accumulation of chromosomal damage that leads to the invasive phenotype. The same stepwise progression was found in patients with single adenomatous polyps and is the base for the adenoma-carcinoma pathway of the majority of colorectal carcinomas (Fig. 1.5).

However, it was soon noted that not all carcinomas are associated with polypoid adenomatous precursors and that there are tumors that are histologically different from the typical adenoma-associated tumors. Following the histologic findings, analysis of chromosomal aberrations showed that many of those tumors are associated with a lack in the DNA repair mechanisms, such as *hMLH1*, *hMSH2*, and *hMSH6*. Those tumors with microsatellite instability are the result of methylation of the normal DNA repair mechanisms that lead to the clonal proliferation of DNA-damaged cell populations that would have otherwise

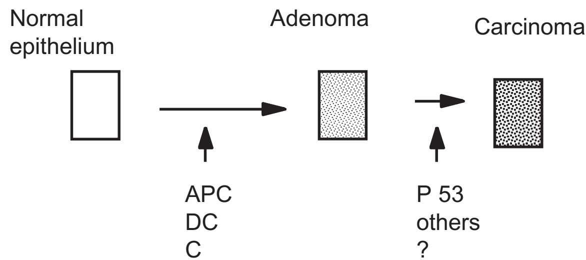


Figure 1.5. Adenoma carcinoma pathway. Stepwise progression of genetic changes from normal colonic mucosa to the development of adenoma and finally invasive carcinoma. Cumulative genetic alterations lead to dysplastic features of cell populations and development of invasive phenotype.

undergone DNA repair. Analysis of these tumors resulted in the finding of other associated tumors in this syndrome, namely uterine and ovarian carcinomas. Analysis of family members can aid in the timely diagnosis of this disease and adequate prophylactic surveillance studies. Additionally, histologic observation that some hyperplastic colonic polyps, which are typically harmless proliferations, are associated with fast proliferationg tumors, lead to the genetic analysis of those lesions. The analysis of these tumors showed that there are in fact specific *BRAF* mutations followed by methylation defects leading to dysplasia and invasive phenotype (Higashidani et al., 2003). Similar lesions in yet another form of colonic carcinoma show hyperplastic-like polyps with histologic changes that are representative of dysplasia. Those serrated adenomas follow a genetic progression different from the above described ways in that those tumors have changes in the *Kras* gene and develop DNA repair defects through the MGMT (O6-methylguanine-DNA-methyltransferase methylation) pathway (Hiyama et al., 1998). It should be of note that in all of these lesions, specific histologic differences between these tumors had been described before implication of molecular pathology and that molecular pathologic data have supported the diagnostic differences and expanded the knowledge of these lesions.

In fact, many systematic molecular pathologic analyses of lesions follow histologic differences made by observant pathologists. Hyperplastic polyps have for the longest time been considered nonneoplastic lesions. Pathologists have described lesions that are very similar to hyperplastic polyps but that have a high malignant potential. Those sessile serrated adenomas show very little histologic difference to hyperplastic polyps, but subtle differences in the glandular structure and the extent of hyperplastic cells along the crypts were a constant feature with polyps associated with malignant tumors.

Molecular pathologic studies not only showed that these polyps are, in fact, different from hyperplastic polyps, they were able to define an entirely new pathway of malignant progression (Makinen, 2007). Whereas many molecular changes have been supporting morphologic observations, other molecular

pathologic changes may precede histologic changes visible through the microscope. In ulcerative colitis, normal appearing colonic mucosa is apparently not affected by the disease process as the histologic features show no significant abnormalities. It has been shown, however, that early genetic alterations that are carried through the stages of low-grade dysplasia, high-grade dysplasia, and invasive carcinoma are already present in histologically normal tissues (Fogt, 1998). This has been shown to be the case in Barrett's esophagus as well, where metaplastic epithelium carries early genetic changes, that is, LOH of the *APC* gene, which are carried through the dysplasia carcinoma progress (Zhuang et al., 1996). Similarly, the observation of blood vessel and clear-cell proliferation in hemagioblastomas in patients with von Hippel Lindau syndrome were thought to represent the proliferation of all genetically altered tissues involved in this tumor (Vortmeyer et al., 1997). Molecular pathologic analysis of these lesions, however, demonstrated clearly that the blood vessels are benign proliferations, which follow the proliferation of the genetically altered clear cells.

## 1.5. MOLECULAR PATHOLOGY IN DRUG DEVELOPMENT

Application of molecular pathology to drug development has been specifically successful in treatment of chronic myelogenic leukemia (CML) and gastrointestinal stromal tumors. The *BCR-ABL* gene in cell-mediated lysis and the *c-kit*-associated fusion genes in gastrointestinal stromal tumor (GIST) result in an altered tyrosine kinase. Whereas the normal tyrosine kinase is inactivated by the binding of its own terminal phosphotyrosine (P) with the activation center, in *c-kit*-positive GIST tumors, this phosphotyrosine cannot bind and inactivate in the genetically altered tyrosine kinase. Here, molecular pathology has proven its place in the pharmaceutical arena with the development of a molecule that can fit into the activation center binding site. Imatinib mesylate (Gleevec) has been introduced as highly potent therapy to suppress the uninhibited activation of the genetically altered tyrosine kinase by binding as an alternative molecule into the inaccessible activation center of the *c-kit*-altered molecule. This treatment has been applied with a high rate of success in chromic myelogenic leukemia and in patients with gastrointestinal stromal tumors that demonstrate similar changes in the tyrosine kinase protein.

The obvious problem that divides the purely academic application of molecular pathology to the pharmaceutical aspect is the way research is performed and supported. The discovery of specific mutations in a given malignant tumor may indicated a pathway of disease development from its earliest beginnings toward the invasive phenotype. This has been shown in colorectal carcinomas, with the *APC* gene on chromosome 5q21 being the first step in the progression from the normal epithelium to dysplastic epithelium. Additional stepwise progression of genetic damage has been shown to lead to advanced malignant and invasive phenotype and, later on, to metastatic phenotype. This kind of progression has been shown to exist in other organ systems as well, such as development of mela-

nomas from dysplastic nevi and the development of breast carcinomas from noninvasive precursor lesions. Unfortunately, there is rarely one single mutation or genetic loss, through whichever mechanism, that can be pinpointed as the truly first step toward tumorigenesis. The *c-kit* story is the famous exception here; the academic research approach in this field, as has been the case when immunohistochemistry was first discovered as a powerful tool to diagnose tumors, is, in many, but clearly not all cases, the categorizations of genetic alterations in given tumors. This may be reflected by the development of a cancer genome atlas that is to be developed to list all genetic alterations, mutations, loss of heterozygosity, and genetic loss found in specific tumors. This is likely not to be helpful in the development of pharmaceutics' progress in cancer treatment as the multitude of genetic alterations will not likely be reversed by acting on one genetic change. Additionally, the listing of genetic alterations is not likely to be followed in the foreseeable future to a cause and reaction analysis. Furthermore, analysis of the main tumor for genetic alterations may not catch the subtle alterations of some tumor clones that will acquire the possibility not only to invade surrounding tissues but the ability to cause metastatic disease, which is what kills the vast majority of cancer patients.

### 1.5.1. Most Important Molecular Pathologic Consideration

The most important aspect when dealing with molecular pathology research is the quality of the tissue in question. When specific tumors are reviewed for molecular pathologic differences or specificities, large numbers of tumors may be obtained from collaborative researchers or from tissue banks. Banked tissues represent small amounts of tumor tissue, usually cut at the working bench, for storage and later research. The labeling of the tumor will be the same as the final pathology diagnosis. Some tissue banks will include quality measures by sectioning at the borders of the tissue and at least review for presence of tumor and/or presence of viable tissue. Not in all cases will the original diagnosis be confirmed on the sections. The tissue blocks received from outside suppliers therefore may or may not contain significant amounts of true tumor and, if necessary, viable tumor, within the specimen. Often a tissue bank specimen will contain mostly necrotic tumor elements; in other cases much of the tissue may represent reactive tissue surrounding tumor material. Before implementing any kind of studies, it is necessary to have the material that is being worked on reviewed by a trained pathologist to ensure that both tumor material and the correct tumor diagnosis is being studied. Depending on the source of the tissues for research, lesions may be solely marked by diagnoses such as the "adenocarcinoma of stomach." It should be noted that such diagnoses will not suffice to characterize the tissue for research sufficiently. One should not combine in a gastric carcinoma study the usual intestinal-type adenocarcinomas with its signet ring cell counterpart, as the cell of origin and the biology of these tumors are quite different. The same is true for many other tumors in different organ systems. Blindly trusting that tumor material is present in the tissue obtained will lead to diluted results, wrong

conclusions, and an increase in the cost of research. When reviewing the results of a study, it is again necessary to ensure that the data are read from malignant tissue and not from benign counterparts or reactive tissues. This may be done by having cases reviewed by pathologists after staining of tissues for immunohistochemical stains, fluorescence in situ hybridization (FISH), or other in situ stains on the slide. When tissues are removed from the slide for microdissection, again, this should be done after a pathologist has marked the tumor areas on the slide in question or a slide of a deeper section. Optimally, microdissection is performed with a trained pathologist reviewing the microdissection itself.

## 1.6. PHARMACEUTICAL DRUG DEVELOPMENT

### 1.6.1. Introduction

The drug development process is a long and arduous road. Nowadays, it takes a compound around 10 years from the time it is discovered to the time it is approved and able to be used by the general population. In addition, it is estimated that the costs associated with developing a new drug are from around $800 million to a billion dollars. And the costs are getting higher and higher each year. It is not our intention to give an exhaustive review of the drug development process in this chapter; rather we want to use this as a general overview of the process. There are a lot of good books published on the subject that can be used if a more thorough knowledge of the subject is wanted. An understanding of the overall process is necessary to better understand the role that molecular pathology can play during this process.

### 1.6.2. Drug Discovery and Development

The whole process of drug discovery and development is summarized in schematic form in Figure 1.6. Each step comprises multiple processes with variable amounts of experimentation associated with them. Several of the steps could be carried out one at a time or simultaneously in order to finish the process in the least amount of time possible. The faster the development of a compound the less cost and greater benefit will be achieved. The role that molecular pathology can play in each of the steps will be expanded in later chapters.

***Target Discovery.*** In order to develop a drug candidate first the clinical condition is identified that needs to be address. Nowadays the question that most people ask themselves is what the unmet medical need is. This need could be in

Figure 1.6. Simplified drug development process.

several fields, cardiovascular, metabolics, immunology, or cancer to name a few. Once the specific medical need is identified the druggability of the process is ascertained. This may require obtaining a better understanding of the disease. This part could be done by reviewing the existing literature in the field and additionally by carrying out experiments to focus in a particular molecule that may play a role in such disease.

Biology is the main driver in this step. Techniques associated with classical biology, like genetics, are employed to find new genes that may lead to new targets. One example of this is in finding drugs in the area of cancer. Cancer is a disease that has been proposed to arise due to mutations in certain genes. It is a stepwise process requiring several mutations to be present for a full-blown expression of the disease. One theory is the two-hit theory developed by Horowitz and colleagues. They postulate that one hit is needed for the cancerous condition to arise and the second hit arises in order for the disease to spread (Horowitz et al., 1990). One of the genes that have been shown to play a role in the cancer pathway is the retinoblastoma gene. This gene plays a major role in the development of a malignant tumor of the retina. The gene is a tumor suppressor, which means that its absence in the cells leads to the development of cancer in the retina. Therefore, this gene may be a target for making a drug. Once this gene has been identified as a key regulator of cancer progression, the biological pathway in which this gene is found could provide several additional targets. If the literature does not provide the pathway genes, then experimental approaches could be utilized. One way that has been used in the past is by identifying other proteins that interact with this gene. One method to do this type of work is by using the genetics of the fruit fly, *Drosophila melanogaster*. Using screens in these animals can lead to the identification of new genes that play a role in cancer. Such a type of screen led to the identification of a new gene, a novel peptidyl prolyl isomerase gene that could be a new target of anticancer agents (Edgar et al., 2005).

Another approach to identify new targets is by looking at proteins already known to be involved in the progression of cancer. An example of this could be the EGFR in colon cancer. This molecule plays a crucial role in the development of this cancer. The EGFR molecule was then chosen to become a target for the development of drugs. Today, there are several EGFR inhibitors on the market. These inhibitors can take several forms; they can be either small-molecules or antibodies. Iressa is an example of the small-molecule inhibitor, which are small molecules synthesized in the laboratory or isolated from nature. The antibodies can be divided into humanized antibodies (a chimeric mouse human antibody that has been modified to elicit no immunological response) and in human antibodies. They have the advantage of being more selective and they tend to have fewer side effects. Erbitux is an example of a humanized antibody, and panitumumab is an example of a fully human antibody. All three of them have been developed as therapeutic agents and are currently available to cancer patients (Mendelsohn and Baselga, 2000; Amado et al., 2008). A consequence of developing these drugs has been the declining mortality of colon cancer over the past few years, demonstrating that this approach to find targets is very useful.

With the advent of the genomics revolution, methodologies that explore the whole human genome are being currently used to find new targets in diseases. Some of those technologies are transcriptional profiling, which will identify all the messenger RNAs (mRNAs) present in a specific sample, proteomics, which will find out the proteins present in the sample of interests, or SNPs (single nucleotide polymorphism) used to find changes in the DNA sequence that may lead to mutant proteins or proteins that are overexpressed.

***Identification of a Drug Candidate.*** Once the protein has been identified to target, we need to find out how to suppress its function. Chemistry, and recently biotechnology, plays the most important role in this arena. There are now two classes of molecules that can be developed to be used as drugs, one is the small inhibitors, chemical entities produced through synthetic chemical reactions or isolated from nature. The other is antibodies, large molecules that are produced in cells through new biotechnology techniques. These molecules are also called biologics to distinguish them from the chemical entities.

Pharmaceutical companies have used the products of chemistry synthesis to discover and refine the small molecules that target specific compounds. One such example is the development of a small molecular inhibitor named dasatinib (Sprycel is its commercial name). This molecule inhibits the *src-abl* oncogene, the main culprit in the disease associated with the Philadelphia chromosome. The presence of such chromosome leads to the development of chronic myeloid leukemia, a devastating type of cancer. The *src-abl* gene was identified as the culprit protein in the translocation whose aberrant function leads to leukemia. Therefore, pharmaceutical companies targeted the protein by making small molecules that were able to knock out the function of the chimeric protein. Chemical synthesis of a battery of related compounds was used to screen for the best candidate to inhibit the *src-abl* oncogene. Upon the production of these compounds, they were further screened for their ability to inhibit other tyrosine kinases and for their antiproliferative effect in human cells and xenographs (Lombardo et al., 2004). Xenographs are human cancerous cells grown in nude mice, mice lacking the immune system. They are a wonderful in vivo model for mimicking human cancers in mice. After the cancers are grown, they are tested for the activities of the compounds. Such models allow for the screening of numerous small-molecule inhibitors before they are even tried in human subjects.

A different way to reduce the function of a target protein is to use antibodies against the molecule that block its properties. This approach has been pioneered by biotech companies and is now widely used. The main advantage of this approach is the increase in specificity due to the use of antibodies. This specificity tends to lead to a decrease in toxicity because there is less off-target effects than using small-molecule inhibitors. While the difference in toxicity and specificity are important, a bigger difference is the manufacturing process required to produce such antibodies. Instead of using chemical synthesis, monoclonal antibodies are produced in cells. The cells are grown in incubators and the antibodies purified from them. The process is not as controlled as the chemical synthesis,

and there are greater variations in lot to lot that need to be carefully monitored. One such example is Erbitux. This molecule is a humanized monoclonal antibody that specifically targets the EGF receptor. It binds to the receptor blocking the signaling from the receptor to its downstream targets (Mendelsohn and Baselga, 2000). This is an important process in colon cancer; blocking this pathway leads to the disappearance of certain types of colon cancers.

Obviously, this is an iterative process. Using either small molecules or antibodies as starting materials, new rounds of compounds or antibodies are tested until the desired characteristics are found. Once that is achieved the compound is now ready for further testing.

***Preclinical Optimization.*** After the identification of a small molecule or an antibody, the next step is to test the compound for toxicities. This testing is administered in vitro and in vivo settings. In vitro refers to the testing conducted in cells in the laboratory, while in vivo refers to the testing carried out in animals. In vivo analysis is required before the drugs are applied to humans. Here the important point to consider is what are the potential side effects or toxicities associated with the compound. Testing is initialized with small animals such as rats and then moved to higher animals such as dogs and chimpanzees. Pathology plays a major role here. Compounds are dosed to find the maximum tolerated dose in each animal species, and pathology is often used as one of the methods to try to find the mechanism of toxicity. After the animals display physical symptoms of side effects, a full physical and pathological examination is carried out. Organs are taken from the animals and examined under the microscope for morphological changes in all the tissues. Here is where classical pathology is doing most of the work in the pharmaceutical development process. Here is also an opportunity to start using molecular pathology. The pathology could give us clues regarding the mechanism of action of the toxicity. This could point to a specific pathway, which can then be investigated using molecular pathology to specifically ask what proteins, mRNA, or DNA are doing in those tissues where the toxicity is observed, thus blurring the lines between classical histopathology and molecular pathology.

If a specific mechanism of action is found for the compound, or a particular side effect is identified, this is a great place to start developing assays that could point them out later during clinical trials. After examining all the experimental results of the in vivo and in vitro experiments and determining what an adequate toxicological profile is, the compound is now ready to start the next phase of human clinical trials.

***Clinical Trials.*** This is the first time that compounds are used in human experimentation. There are four phases of clinical trials: phase I, phase II, phase III, and phase IV.

Phase I is carried out to determined the metabolism and pharmacologic actions of drugs in humans. (For a thorough review of the clinical process see http://clinicaltrials.gov.) These are medical trials that enroll a small number of

patients and try to demonstrate that the drug does not have significant adverse events in humans. The way these trials are normally designed is to start dosing human subjects with the smallest amount of the drug possible and then raise the amount while carefully monitoring the patients. The starting dose is obtained by taken into consideration the preclinical results with animal experimentation. The equivalent starting dose is the dose that when extrapolated from animal results in the lowest dose at which no side effects are expected and efficacy is expected. If there are toxicities associated with the compound, an even smaller equivalent dose is used to start dosing human patients. This dose is progressively increased, keeping the patients under conditions in which the possible side effects can be monitored and brought under control. Obviously, the importance of safety cannot be underestimated in this process and priority has to be given to patients' well-being. The side effects could expand the whole rage of illnesses: from rash to vomiting, hepatocellular abnormalities, or even cardiovascular abnormalities. Some of these could be easily monitored, like checking for specific enzymes in the blood that are associated with tissue disease, such as elevated levels of lactate dehydrogenase, or LDH. The threshold for accepting side effects varies with each indication. Depending on the disease in question, the safety barrier associated with a drug may be put at different levels. For example, if the indication is for a population of terminally ill patients, like those associated with some types of metastatic cancer, higher tolerance for abnormal side effects, such as high-grade rash, loss of hair, or vomiting, may be allowed. While if on the other hand the drug is for an indication in which there is not immediate death, such as ulcerative colities or rehumatoid arthritis, then the safety barrier is higher.

Another goal of the phase I trials is to start gaining early data on the effectiveness of the compound. While these trials tend to be small in number, raging from 20 to 100 patients, and the results may not be statistically significant, they may provide some guidance for a subpopulation of patients to conduct the following phases of clinical research.

Phase II is started after concluding phase I. These are controlled clinical studies that start to look at the effectiveness of the drug. Part of the emphasis is in identifying the right population that the drug is going to target and to find the right dose for the patients. In this phase safety is still being evaluated, side effects are continually monitored, and the risks keep been assessed. A larger number of patients are used to try to get statistically significant numbers to answer questions of effectiveness. Lately, it is very common to include as a secondary goal in these trials some biomarkers. Biomarkers are surrogate measurements that may indicate that the drug is having an effect. There are several types of biomarkers. Those showing changes that correlate with drug dose are named pharmacodymanic markers, and others that may select which population of patients is more likely to respond to the treatment are called predictive biomarkers. Nowadays, there is a lot of emphasis and research in this area. Even the Food and Drug Administration (FDA) is getting in the act, and it is issuing new guidelines regarding the use of biomarkers in clinical trials. One nice effect of utilizing these markers is that it could find faster the patients most likely to improve with treatment, with lesser

side effects, use a smaller number of patients and therefore lower the cost of developing the drug, which in turn will reduce the overall cost of drugs in the market. Thus, the use of biomarkers may benefit both the patients and the pharmaceutical companies. Further use of biomarkers is explained in several of the chapters in this book, and the demonstration of the use of biomarkers in the drug development process is shown in more detailed in Chapters 3 and 6, where the authors show several examples of biomarkers for both predictive and pharmacodymanic uses in early clinical trials.

Once a phase II is completed the process moves to phase III. Here, the trials are with an even greater number of patients with the goal of getting at the effectiveness of the drug. At the same time, additional information is gathered regarding the side effects, and an overall picture of the risks and benefits is obtained. These will serve as the bases for the product description that appears in the label of the drug. Each new drug carries a label. In it, the intended use of the drug, the population to which the drug is targeted for, and the known side effects are enumerated. This label will contain the basic information needed by physicians to prescribe it to their patients.

***Approval of a Drug.*** After completion of the three first phases, the data is presented to the medical authorities for approval of the drug. In the United States, the FDA is the governmental body entrusted with deciding the safety and efficacy of all new drugs. In Europe, the European Agency for the Evaluation of Medical Products (EMEA) fulfills the same role for the European Union. For the rest of the world each country has its own rules and regulations. Some of those countries will first ask that a drug be approved by the EMEA or the FDA before they even look at the application. Other countries, like Japan, require that the clinical trials may be done in patients that are genetically similar to the population present in their respective countries. This is to prevent the appearance of new side effects in the native population that may be due to a different genetic background from the subjects that were used for the initial clinical trial. Interestingly, the agencies may approve, request additional information, or deny a drug application. Approval from one agency does not automatically mean the other agencies may conclude the same. Additional information may be required for each agency, such as the running of new clinical trials to specifically target an observed anomaly in a subpopulation of the patients' that was observed previously. An example could be to repeat a clinical trial with an expanded number of patients where a specific cardiovascular risk is examined.

***Life-Cycle Management.*** After a drug has been approved for a specific disease, other clinical trials may be started to look for additional indications in the population. These clinical trials are referred to as phase IV. As before in the other phases of the process, information regarding safety and side effects is of vital importance. It is at this point of the process that doctors can start using the medication in other areas as appropriate. For example, in the case of Erbitux, it was first approved for use in colon cancer patients as a single