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CURRENT TECHNOLOGY IN CANCER RESEARCH AND TREATMENT

Use of radionuclides in cancer research and treatment

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Abstract Cancer occurs as a result of misregulation of cell growth, which appears to be a consequence of alteration in the function of oncogenes and tumour suppressor genes. Ionising radiation has been used, since the discovery of X-rays in 1896 by Roentgen, both in cancer research and treatment of the disease. The main purpose of cancer research is to understand the molecular alterations involved in the development and progression of the disease in order to improve diagnosis and develop personalised therapies, by focusing on the features of the tumoral cell and the biological events associated to carcinogenesis. Radioisotopic techniques have been used routinely for *in vitro* research in the molecular and cellular biology of cancer for more than 20 years and are in the process of being substituted by alternative non-radioactive techniques. However *in vivo* techniques such as irradiation of cells in culture and/or experimental animal models and radioactive labelling are in development, due in part to advances in molecular imaging technologies. The objective of this review is to analyse in an integrative way the applications of ionising radiation in cancer research and therapy. It had been divided into two parts. The first one will approach the techniques applied to cancer research and the second will summarise how ionising radiation is applied to the treatment of neoplastic disease.

Keywords Cancer · Radioactive technique · Molecular imaging · Research · Diagnostic

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Introduction

Cancer is a systemic disease [1] that results from changes in the interaction between oncogenes and tumour suppressor genes, resulting in the loss of cell growth control [2], since only tumoral cells have unlimited replicative capacity and invade proximal and distant tissues [3].

Even though many biological alterations were discovered in tumoral cells more than 70 years ago [1], there are still may unresolved issues [4] that need to be clarified in order to gain a deep understanding of the molecular mechanisms involved in the generation and progression of cancer. This will help to improve diagnosis and prognosis, and develop new therapeutic strategies. Today, thanks to advances in molecular and cellular biology techniques, numerous specific molecular markers have been identified that could help in the development of personalised treatments [5].

Since Roentgen discovered X-rays in 1896 [6], these have been used in the diagnosis and treatment of cancer, and ionising radiation and biology have evolved together in the research of neoplastic disease, as in clinical practice. Radioisotopic *in vitro* techniques have been used routinely in molecular and cellular biology research laboratories devoted to cancer research. However, in recent years these have been replaced by alternative non-radioactive techniques, such as colorimetric- and fluorometric-based techniques that allow the same precision as radioactive ones but reduce the risks. Undoubtedly, enormous advances have been made in the development and application of molecular imaging technologies, mainly in *in vivo* techniques. The important contribution of this technology is based on the possibility of studying molecular and cellular events that several decades ago could only be approached *in vitro* and now can be performed in living organisms [4], giving information of high impact on the diagnosis, treatment and prevention of the disease [7, 8].

Table 1 *In vitro* radioisotopic techniques

Technique	Radioisotope	Sub-technique	References
Enzymatical assays	^{14}C	CAT assay	12
	^{32}P	TRAP assay	12
Labelling of cell culture	^{3}H	Cell proliferation	13, 14
	^{35}S	Protein synthesis	13, 15
Hybridisation	^{32}P	Southern, Northern	12, 15–17
	^{35}S	*In situ* hybridisation	18
Characterisation of DNA	^{32}P	Footprinting	18
	^{32}P	Band shift	12, 15
	^{32}P	Amplification of fragments	12
Binding assays	^{3}H, ^{125}I	Receptor identification	19
In vitro transcription	^{35}S	mRNA detection	15, 18
In vitro translation	^{32}P, ^{35}S	Protein synthesis	18

CAT, chloramphenicol acetyltransferase; *TRAP*, telomeric repeat amplification protocol; *RFLP*, restriction fragment length polymorphism; *AFLP*, amplification fragment length by PCR

The objective of this review is to present a simultaneous and integrated analysis of the applications of ionising radiation in research and therapy in cancer. The review has been divided into two parts. The first one presents the state of the art technology involving ionising radiation in cancer research, mainly focused in molecular imaging, taking into consideration its relevance for the neoplastic disease. The second one will present a general view of the different therapeutic treatments based on the use of radiation, both in use and in the development phase.

The main goal in cancer research is to understand the molecular events in all neoplastic diseases [9] that allow the development of cancer and try to correct or control them. In the beginning of the 21st century medicine has adopted a molecular approach in the diagnosis and treatment of cancer [10], therefore conceptually an efficient cancer treatment should eliminate the biological event that originated the disease [11]. At this time multiple research approaches are in development, focused on the study of the features of the tumoral cell (metabolism, signalling pathways and associated receptors that favour proliferation and prevent apoptosis in tumoral cells); alteration in gene expression during the evolution of the disease, epigenetic and DNA repair; biological events associated with cancer (cell proliferation, apoptosis and hypoxia) and molecular alterations associated with tumour progression (angiogenesis and metastasis).

In vitro radioactive techniques

Molecular and cellular *in vitro* radioisotopic techniques have been used for many years on a routine basis in basic cancer research, mainly due to their sensitivity and accuracy. Although some of them are being replaced by non-radioactive techniques, these do not always allow the final objective of the study to be accomplished. Therefore radioactive-based techniques continue to be used. Table 1 contains these *in vitro* techniques used in the field of molecular and cellular biology cancer research.

The techniques in Table 1 are used to study, among other things, different features of tumoral cells: gene expression [12–18], DNA repair mechanisms [13] and signalling pathways and receptor expression [12, 19]; and biological events associated to cancer: cell proliferation [12–14], invasiveness and apoptosis [15] and identification of tumour markers (RFLP, AFLP) [12]. On the other hand, *in vitro* techniques are also used to confirm data that have been obtained *in vivo*; PET signal *in vivo* can be quantitatively linked with gene expression levels directly obtained *in vitro* [14, 20–23].

In vivo radioactive techniques

In vivo assays are necessary to translate results obtained in basic research to clinical practice. Therefore, these techniques, in contrast to what happen with *in vivo* ones, by using animal models are rapidly growing, mainly due to the growing development of molecular imaging techniques and the advances in gene therapy.

In vivo radioactive techniques can be subdivided into two groups, based on the radioactive source used and the objective of the study:

a) irradiation techniques of cell culture and experimental animal models and

b) radioactive labelling techniques.

Irradiation techniques of cell culture and experimental animal models

These techniques use biological irradiators equipped with sealed sources, generally ^{137}Cs, of high activity (30–37 TBq). These applications are used to measure effects induced by radiation in different external conditions.

One of the main objectives of irradiation of animal models is to gain knowledge on the effect of ionising radiation to implement radiotherapy treatments. High doses of radiation can affect tumoral cells as well as healthy tissues next to the irradiated area.

A system of microradiotherapy has been developed for small animals to allow simulation of irradiation applied to patients with different radiotherapy treatments [24], thus allowing the study of molecular changes induced by different radiation doses. Coleman [7] described the bystander effects in which the non-irradiated cells suffer biochemical changes, provoked by molecules released by irradiated cells, that can be transmitted thorough gap junctions at the membrane or to the extracellular media. Considering the relevance that these effects can have in patients subjected to radiotherapy, the different signalling pathways involved are being studied [25] as well as the different agents that can potentiate this effect [26].

Another aspect under study is the sensitivity of the tumoral cell [27] and the effect induced by radioprotective agents used simultaneously in radiotherapy treatments [28].

On the other hand, experimental irradiation is being applied to measure the DNA damage induced and to examine the mechanisms involved in repairing this damage by studying the critical role that cancer stem cells play [29] and its function in different radiotherapy treatments [30].

Radioactive labelling techniques

In this approach, different non-sealed sources such as the ones used in *in vitro* techniques and labelled with different radionuclides are given to the organisms under study. The applications of these labelling techniques include functional studies (non-included in this review), in gene therapy and molecular imaging.

Techniques of molecular imaging

The term molecular imaging was created in 1990. The technique results from the combination of molecular and cellular biology, chemistry and electronic imaging and is defined as the *in vivo* characterisation and measurement of biological processes at cellular and molecular levels [31].

There are different types of molecular imaging and the main difference between them is in terms of spatial and temporal resolution and sensitivity [32]. They can be subdivided into two large groups: structural techniques such as computed tomography (CT), magnetic resonance imaging (MRI) and ultrasonography (US), and functional techniques such as single-photon emission computed tomography (SPECT) and positron emission tomography (PET).

Structural or anatomical techniques such as CT give anatomical information in a qualitative manner [33], allowing the detection of the tumour, measurement of the therapeutic response and determination of relapses based on features such as size and localisation of the tumour. However, these techniques by themselves do not allow the characterisation of the neoplastic disease because they do not give information about the molecular and physiological characteristics of the tumour [6]. It is also known that molecular functional changes precede anatomical ones during the progression of the disease [16]. Nowadays it is possible to obtain this functional information thanks to the growing development of emission techniques such as SPECT or PET in which a radiotracer is administered to the organism. This gives molecular and functional information about the tissue and allows characterisation of biological properties of the tumour [6].

As well as its important and numerous applications in the clinic, molecular imaging is being used every day more as a research technique in cancer since it allows *in vivo* visualisation and measurement, in a non-invasive manner, of different biochemical, physiological and physiopathological processes at molecular and cellular levels, which allow distinction between healthy and tumoral tissue. These techniques have high sensitivity in the nano–pico mole range [32, 34], allowing the study of molecular alterations, the basis of the disease [31]. It was already predicted at the beginning of the 21st century that molecular imaging was going to be one of the most important areas in oncology [32].

SPECT was initially developed for use in humans, but it was adapted to visualise small animals with high spatial resolution for basic and translational research [35].

The development of PET began in the 1950s but the first equipment for clinical use was developed by Michael Ter-Pogossian and colleagues in the 1970s [6]. Massoud and Gambhir [36] have published an extensive review of molecular imaging techniques, making a comparative analysis in terms of spatial and temporal resolution and between the sensitivity of the different techniques, also considering the advantages and disadvantages of each of them, numbering the different probes and suggesting applications to research in cancer. Other reviews of the application of molecular imaging to cancer research can be found in Refs. [6, 10, 33, 37–40].

The probes designed for molecular imaging are a biological tool that reveal the molecular bases of normal and pathological processes [1]. They can be used both for therapeutics and for diagnostics [39]. The process of developing and validating new radiotracers has been described by Blasberg [41]. More than 500 probes have been synthesised for molecular imaging [42] and among them are different enzymes, hormones and antibodies, many of them labelled with different radionuclides and well developed to visualise different biological events in cancer [41].

The radioisotopes used for PET studies such as ^{11}C and 18F cannot substitute radioactive carbon and hydrogen in any biological molecule [33], allowing great versatility in *in vivo* biochemical studies [43]. The synthesis of radiotracers labelled with ^{11}C has several limitations [44]. The short period of semi-disintegration of ^{11}C (20.4 min)

means rapid preparation and purification of the molecules labelled with this nucleotide are required.

No imaging technique can provide all the information of the tumoral profile [6] and the solution is to use functional techniques in conjunction with structural techniques in a synergic manner. Multimodal imaging has been developed [36] and it registers and combines images acquired using different imaging techniques such as PET/CT. The fusion of these images simultaneously maximises the morphological and functional information. Different studies that allow validation of PET/CT [45] indicate that multimodal imaging increases accuracy in diagnosis when compared with PET alone [10].

In recent years the different molecular imaging techniques have been adapted to small animal experimentation. MicroSPECT [46] and microPET have been developed with a resolution of 1.8 mm^3 [32]. Meikle et al. [35] have recently reviewed the state of the art of microSPECT development. Different studies done in small animal models have constituted a bridge linking *in vitro* data and its translation to clinical applications [47].

The early detection and precise staging of small tumours gives important data to obtain good results in the treatment of the disease [6]. Weissleder suggested that the diagnosis of stage I cancer is associated with 90% survival in 5 years [38]. Several authors have also pointed out how important early and differential diagnosis is for the evolution of cancer and the prognosis of the disease [3, 16, 31, 50].

Metabolism of a tumoral cell

In 1924, the German biochemist Otto Warburg and colleagues published their observations on the metabolism of the tumoral cell [1]. Tumour cells show different metabolism alterations as they require more nutrients (glucose and amino acids) in order to maintain the increased energetic level for the synthesis of proteins required for DNA replication due to an increase in cell proliferation. Besides, tumoral transformation is associated with specific alterations of choline metabolism considering the increase in phospholipid synthesis in the cellular membrane associated with tumour progression [48]. This is the reason why in many tumoral cells choline, phosphocholine and phosphoethanolamine levels are increased. Jager et al. [37] have reviewed the methods currently available to visualise metabolic processes in tumoral cells in preclinical models and describe different types or radiotracers and their applications in different types of cancer.

Alterations in glucose metabolism are very common in tumoral cells [1]. The glucose turnover is different between healthy and tumoral tissues and is inversely related to the degree of cell differentiation in tumoral cells. The glucose analogue 2-[^{18}F]fluoro-2-deoxy-D-Glucose (FDG) was tested in humans in 1976 [33]. FDG-PET allows the measurement of increased glucose metabolism, associated with changes in glycolytic enzymes and overexpression of glucose transporters [49]. This alteration is a clear indicator of malignancy [33] and allows differentiation of malignant vs. healthy tissues. FDG has been confirmed as a marker for metabolic activity in the tumoral cell by different authors [10, 33, 42, 48, 49].

Phelps [1] has reviewed the applications of FDG for diagnosis, staging of the most common types of cancer and indicating advantages and disadvantages of the technique in each tumour. The relevance of this technique is widely described in the literature [19, 38, 39, 41, 50]. Besides the benefit of the use of FDG-PET to an accurate diagnosis of cancer, it is also valid for the detection of relapses with 80–90% accuracy [33].

However, FDG-PET shows some limitations in measuring tumoral metabolism. Tumours that grow slowly, such as prostate and thyroid, have low glucidic metabolism [33], showing lower incorporation of FDG than tumours of fast growth [6]. On the other hand, the incorporation of glucose is not specific to tumoral tissue since healthy tissues such as salivary glands [6] and some pathological situations (infection of inflammation) [10] also uptake FDG and could give false positives in oncological studies.

Receptors and signalling pathways

Cancer appears as a result of the accumulation of alterations in genes that are constitutive critical components in the systems of signal transmission and whose alteration results in uncontrolled proliferation [2]. Different tools have been described to monitor intracellular cascades involved in cell transformation [3, 4, 51].

Proliferation of certain tumours is controlled in part by hormones or growth factors that are bound to specific receptors (either at the membrane or intracellular) by forming complexes involved in different routes of signalling that regulate both proliferation and cell death [6, 10]. Table 2 reflects different imaging agents proposed to visualise receptors associated with cancer.

Epidermal growth factor (EGF)

Epidermal growth factor (EGF) and its receptor EGFr are frequently overexpressed in the tumoral cell membranes of epithelial origin [52]. EGFr is a transmembrane glycoprotein involved in the activation of different processes associated with tumorigenesis. Its overexpression has been related to an increased metastatic potential and bad prognosis [6].

The first labelled markers proposed to visualise EGFr using PET were [^{18}F]FMLO (reversible inhibitor) and [^{11}C]MLO3 (irreversible inhibitor). Although these interacted properly with the receptor and showed good inhibitory ac-

Table 2 Imaging agents to visualise receptors related to cancer

Receptor	Image agent	Technique	References
EGFr	AntiEGFr Abs	SPECT	6, 10
	[^{18}F]FMLO	PET	52
	[^{11}C]MLO3	PET	52
Oestrogen receptor	[^{18}F]progesterone	PET	6
	[^{18}F]FES	PET	1, 6, 10, 33, 39, 43, 52
Androgen receptor	[^{18}F]FDHT	PET	6, 10
SSTR2	[^{111}I]pentetreotide	SPECT	33, 36, 54
	[^{68}Ga]DOTATOC	PET	10, 33, 52
	[^{18}F]TOCA	PET	33

FES, [^{18}F]fluoroestradiol; FDHT, fluoro-5α-dihydrotestosterone; [^{68}Ga]DOTATOC, [^{68}Ga]D-Phel-Tyr3-octreotide; [^{18}F]TOCA, Gluc-Lys (^{18}F-fluoropropionyl-TOCA)

tivity, their possible pharmacological use has been rejected, due to its fast *in vivo* degradation [52]. The use of anti-EGF antibodies to SPECT studies, with better image quality than the EGF analogues, has also been suggested [6].

Estrogen receptor (ER)

Most breast tumours are hormone-dependent [6]. For this reason, several techniques have been developed to visualise the ER. Initially, the use of [^{18}F]progesterone was suggested, although it has since been rejected because of its low sensitivity [6]. [^{18}F]fluoroestradiol-17ß (FES) has been proposed as a possible effective marker to visualise ERs in breast tumours [6, 10, 33, 52]. There is a good correlation between the incorporation of FES and ER concentration [39, 43], allowing identification between primary tumours and metastatic nodules regarding their expression levels [6]. ER status is one of the better established predictive markers in oncology [39], representing an important prognostic index in patients with breast tumours [43].

Androgen receptor

Many prostate tumours express androgen receptor, which is predictive of the hormonal therapy response [6]. The first images studied from this receptor were developed with [^{18}F]fluoro-5α-dihydrotestosterone (FDHT) [6, 10], an analogue of the sexual male hormone dihydrotestosterone. However, its clinical use is limited by its low incorporation in the prostate tumour tissue and its high activity in normal tissue [6].

Other receptors overexpressed in tumours

A variety of receptors for peptides, similar to somatostatin, gastrin, bombesine, neurotensin [52] or neuropeptide Y (NPY) [53], are overexpressed in different tumours, like breast, pancreas, small-cell lung cancer, prostate and thyroid [9]. Several labelled probes have been suggested to detect the overexpression of those receptors [9]. However, only the somatostatin receptor (SSTR) [52], overexpressed in neuroendocrine tumours (NET), has been completely studied.

Different radiotracers derived from the octreotide, a somatostatin analogue protein, with high affinity to the subtype 2 SSTR2 have been developed. The most frequently used ligand for SPECT studies is the [^{111}In]pentetreotide [33, 54]. Other labelled ligands to visualise SSTR2 by using PET are [^{68}Ga]D-Phel-Tyr3-octreotide ([^{68}Ga]DOTATOC) [33, 52] and Gluc-Lys ([^{18}F]fluoropropionyl-TOCA) [33]. This radiotracer has considerably increased the quality of PET imaging [55], allowing good results to be obtained in preclinical and clinical studies in many tumour types, particularly in NET [55]. On the other hand, the use of NPY-labelled analogues has been proposed for diagnosis applications in different tumours [53].

Cellular proliferation

Uncontrolled cellular proliferation, as has been indicated previously, is one of the main characteristics of malignant tumours, considered as a marker for tumour malignancy [6]. Incorporation of FDG into tumour cells is not a specific marker of cellular proliferation or tumour growth because other healthy or pathologic tissues can also incorporate it. Therefore, it has been a priority to develop specific markers for tumour cellular proliferation. The visualisation of cellular proliferation can be done by analysing the incorporation and metabolism of nucleosides, used in DNA synthesis, labelled with ^{11}C or ^{124}I in PET studies [38, 48].

More than 40 years since its development, [^{3}H]thymidine is still a good standard to measure cellular proliferation *in vivo* [49]. The first radiotracer of cellular proliferation studied and visualised *in vivo* was [^{11}C]thymidine, synthesised by Christman and Brookhaven [39], and it was proposed to visualise cellular proliferation by other authors [49, 52] but it is used less every day because of its fast catabolism and metabolic instability [6, 52]. Several halo-

genated analogues of thymidine have been developed, such as [^{131}I]- and [^{124}I]deoxyuridine [6] and [^{77}Br]deoxyuridine [56]. Wagner et al. [49] have shown that *in vivo* incorporation of [^{18}F]fluorodeoxyuridine (FudR) is correlated with cellular proliferation in an animal model for pancreatic cancer. However its metabolic instability limits its clinical use [49, 57] and occurs with [^{11}C]thymidine [49]. This inconvenience has been overcome with the development of fluorinated tracers that show better metabolic stability [52]. One of the nucleoside analogues better studied to visualise cellular proliferation is [^{18}F]3'-deoxy-3'-fluorothymidine (FLT), first proposed for this purpose by Shields et al. in 1998 [39]. Wagner et al. evaluated FLT as a PET tracer to visualise cellular proliferation [49]. Their results indicated that FLT represents a new non-invasive tool for the diagnosis and staging of malignant lymphoma by measuring cell proliferation in these tumours. However, it underestimates proliferation in patients with aggressive tumours. That is why more studies are necessary to establish the advantages and disadvantages of the use of FLT as a tracer of proliferation.

FLT is being validated to visualise cell proliferation in animals and patients [52] and preliminary data indicate it is a good marker for cell proliferation [33], its incorporation has been correlated with pathological cell proliferation in many tumours [58] and it has been used in the clinic in patients with non-small-cell lung carcinoma (NSCLC), colorectal cancer [6], and in breast cancer, gliomas and oesophagic carcinoma with satisfactory results [39].

In some scenarios FLT-PET has demonstrated higher specificity for cancer than FDG-PET, which can show false positives because it is incorporated into areas of inflammation and infection [58], as previously indicated. FDG can be found both in tumoral and inflammation tissues but FLT is incorporated only in tumoral tissues [10]. Taking into consideration the low incorporation and high background of FLT-PET in liver and bone marrow, FLT could be considered a substitute for FDG in cancer staging and in other cases used in combination with it to give complementary information for the diagnosis [58].

Another evaluated pyrimidine, ^{18}F-54'(-2'-deoxy-2'-fluoro-beta-D-arabinofuranosyl)-thymine (FMAU), showed better incorporation in DNA than FLT, but there are no conclusive results to confirm the image of proliferation [52].

A different suggested strategy as an indirect measurement of cellular proliferation is the measurement of protein synthesis using labelled aminoacids. The best studied is [^{11}C]methyl-methionine ([^{11}C]MET) [6, 39], chemically identical to non-radioactive methionine, and therefore metabolised by the cell and incorporated into proteins [33]. One of the advantages of MET over FDG is that MET can discriminate between tumoral cells and those subjected to inflammation because inflammatory cells have a lower protein metabolism than that of glucose. [6]. But MET is not an accurate marker for cellular proliferation in all tumours. Incorporation of MET correlates well with cellular proliferation in brain tumours and NSCLC but not in head and neck squamous cell carcinoma (HNSCC) and lymphomas [6]. Besides, its short period of disintegration of ^{11}C limits its use [33].

Other aminoacid analogues of tyroxine are promising for the study of cell proliferation: L-3-yodo-α-methyltyroxine (IMT) [6], O-(2-[^{18}F]fluoroethyl) L-tyroxine (FET) [6, 52] and L-3-548F-fluoro-α-methyl-tyroxine (FMT) [6, 33]. [^{18}F]6-fluorodihydroxyphenylalanine ([^{18}F]-FDOPA), an analogue of L-DOPA, is accumulated in dopaminergic neurons, allowing visualisation of gliomas when comparing with FDG and better results in the clinic [33]. Choline is another indicator of cellular proliferation that increases its levels in prostate tumours [48], as previously indicated. Many tumours express high levels of cholinekinase I, the enzyme that phosphorylates choline. The accumulation of phosphorylated choline indicates high levels of cell proliferation [10]. The use of choline, labelled with ^{18}F or ^{11}C, has been proposed to visualise cell proliferation [6, 16]. [^{18}F]fluorocholine ([^{18}F]FCh), [^{18}F]fluoroethylcholine ([^{18}F]FECh) and [^{11}C]choline ([^{11}C]Ch) [10] have been measured as substrates of choline kinase in order to visualise cell proliferation in prostate cancer [10, 52].

Table 3 contains a summary of molecular probes useful to visualise different biological events associated with cancer.

Apoptosis

Apoptosis, or programmed cell death, was described for the first time in 1972, by Kerr et al. [48]. It is a process typical of eukaryotes that is necessary for several physiological processes [10]. It is negatively regulated in cancer, therefore playing an important role in the maintenance of the disease [60] and in the response to radiotherapy. For this reason there is a growing interest in investigating it as a marker of cellular radiosensitivity [6] and prognosis [59].

Annexin V is a protein that binds phosphatidylserine (PS) in the membrane of an apoptotic cell. Once labelled with ^{99m}Tc, ^{124}I or ^{18}F it can be used in SPECT or PET studies [10] and it has been proposed as a promising apoptosis marker [33, 38]. In preclinical models of NSCLC, lymphomas, breast cancer and sarcomas an accumulation of [^{99m}Tc]annexin V has been described in apoptotic tissues after treatment with radiotherapy [6]. In other studies done in order to visualise apoptosis, it was attempted to label inhibitors of caspases, but they did not prove to be useful due to the low capacity of incorporation [52].

Hypoxia

In a solid tumour, vasculature is defective and there are frequently areas of hypoxia [48], like in NSCLC and HNSCC where areas with 80% hypoxia have been found [6]. Hypoxia is an important factor in oncology [60] and it can be a limiting factor for patients subjected to radiotherapy treat-

Table 3 Examples of molecular probes to visualise biological events associated with cancer

Biological event	Molecular probe	Technique	References
Cellular proliferation	[^{11}C]thymidine	PET	6, 49, 51, 52
	FLT	PET	6, 10, 33, 36, 39
	FMAU	PET	39, 52
	IMT	PET	6
	FET	PET	6, 52
	FMT	PET	6, 33
	[^{11}C]MET	PET	6, 33, 39
	[^{18}F]DOPA	PET	33
	[^{18}F]FCh	PET	10, 52
	[^{18}F]FCCh	PET	10, 52
	[^{11}C]Ch	PET	10, 52
Apoptosis	[^{99}Tc]annexin V	SPECT	6, 10, 33, 38
	[^{124}I]annexin V	SPECT	6, 10, 33, 38
Hypoxia	FMISO	PET	62, 7
	FETNIM	PET	52
	FETA	PET	6
	EF5	PET	6, 33
	^{123}I/^{18}FFAZA	PET	6, 52
	Cu-ATMS	PET	39, 52
Angiogenesis	[^{18}F]galacto-RGD	PET	6, 33, 52
	RGD-mimetic^{18}FGBHO	PET	6, 52
	AntiED-B Abs fragment-	PET	6, 52
	[^{124}I]HuMV833 Abs	PET	6, 52
	^{125}I, ^{153}S, ^{99m}TcVG76e	PET	6
	[^{99}Tc]EC-endostatin	PET	6

FLT, fluorothymidine; *FMAU*, [^{18}F]54'(-2'-deoxy-2'-fluoro-beta-D-arabinofuranosyl)-thymine; *MET*, methyl-methionine; *IMT*, L-3-iodo-a-methyltyrosine;
FET, fluoroethyl-L-tyrosine; *FMT*, [^{18}F]α-methyl-tyrosine; [^{18}F]-FDOPA, [^{18}F]6-fluorodihydroxyphenylalanine; [^{18}F]-FCh [^{18}F]fluorocholine; [^{18}F]-FECh), [^{18}F]fluoroethylcoline; [^{11}C]Ch, [^{11}C]choline; *FMISO*, fluoromisonidazole; *FETNIM*, fluoroerythronitroimidazole; *FETA*, fluoroetanidazole; EF5, 2-(2-nitro-1[H]-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)-acetamide; *IAZA/FAZA*, iodo/fluoroazomycin arabinoside; *Cu-ATSM*, Cu(II)-diacetyl-bis(N(4)-methylthiosemicarbazone); *RGD*, Arg-Gly-Asp sequence; *EC*, ethylenedicysteine; *Abs*, antibody

ments [6, 33, 54] because hypoxic tumoral cells are 2–3-fold more resistant than normal cells [52] due to the low tension of oxygen. Considering the relevance of this biological process in tumour progression and in response to radiotherapy, there is growing interest in developing methods to visualise and quantify this. Measurement of oxygen in the tissue using SPECT and PET [61] has been performed in animal models, but two groups of markers to visualise hypoxia have been proposed in molecular imaging.

One of these labelled compounds is 2-nitroimidazole and it was proposed 20 years ago [6]. Fluoromisonidazoles are lipophilic compounds that bind to intracellular proteins when the concentration of oxygen is lower than 20 mmHg [33]. [^{18}F]fluoromisonidazole (FMISO) for PET is one of the more intensively studied radiotracers for hypoxia: it selectively accumulates in hypoxic tissue and was used as a prognostic marker for head and neck cancer after treatment with radiotherapy [62]. However its clinical use is limited by its low incorporation into hypoxic tissue [6]. A new generation of nitroimidazoles are being synthesised and used in animal models: [^{18}F]fluouoerythronitroimidazole (FETNIM) [52], fluoroetanidazole (FETA) and 2-(2-nitro-2 [H]-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)-acetamide (EF5) [6]. In animal models EF5 has allowed the identification of hypoxic areas and the evaluation of responses to radiotherapy in gliomas and head and neck cancer [33].

Other potential hypoxic markers have been suggested: iodinated azomycin arabinosides (IAZA, IAZGP, IAZXP), ligands for SPECT [6] and [^{18}F]fluoroazomycin arabinoside (FAZA) for PET [6, 52]. [^{60,62,64}Cu]diacetyl-bis-N4-methylthiosemicarbazone (Cu-ATSM) was discovered as a hypoxic marker and its use is under study [39] since its mechanism of retention is not well understood [52].

Tumour progression: angiogenesis and metastasis

Tumour progression is a sequential process in cancer that involves different biological events including angiogenesis and induction of metastasis, both important determinants of the evolution and prognosis of cancer.

Angiogenesis

In the middle of the last century, Thomlinson and Gray, by studying tumour hypoxia, established the bases to in-

vestigate in tumoral angiogenesis [48]. Later, Folkman identified angiogenesis as a potential tumour target [48]. This biological event has been recognised as an essential process in tumour progression and metastasis in human solid tumours [10, 63]. The development of radiotracers to visualise apoptosis has been focused on inhibitors of matrix metalloproteinases (MMPs), which facilitate invasion of adjacent tissues, peptide or non-peptide antagonists of integrins (mainly αvß3 integrin), single-chain antifibronectin antibody fragments [6] and radiotracers directed at vascular endothelial growth factor (VEGF) [52].

Overexpression of MMPs has been correlated with an increase in aggressiveness and metastatic potential in many tumours. The first studies oriented to visualise angiogenesis were performed by using inhibitors of MMPs, for example in PET using CGS27023A and SAV03, inhibitors of MMP3 and MMP2, respectively, and labelled with ^{11}C, ^{18}F and ^{64}Cu in breast cancer [6]. αvß3 integrin is overexpressed in tumoral vasculature in many solid tumours, indicating an aggressive phenotype [64]. This integrin binds peptides that contain the aminoacid sequence Arg-Gly-Asp (RGD) [33, 54], present in ECM proteins (selectively expressed extracellular matrix). These compounds have been labelled with radionuclides SPECT as ^{99m}Tc, ^{111}In, ^{90}Y [6] and radionuclide PET as ^{18}F [33], which bind integrins in angiogenic endothelial cells, allowing its visualisation. The best results in visualising αvß3 integrin have been obtained with ^{18}F-galacto-RGD and RGD-mimetic ^{18}F-GBHO [6]. The incorporation of ^{18}F-galacto-RGD in solid tumours has been correlated with the expression of αvß3 integrin in the tumoral cells and is associated with higher metastatic potential [33]. This ligand has been evaluated with good results in clinical studies in patients with melanomas, sarcomas, head and neck cancer, gliomas and breast cancer [52].

Fibronectin is an EMC protein involved in different processes such as cell migration and is highly expressed in tumoral tissue, therefore it has been proposed as a potential angiogenesis marker. The domain ED-B of fibronectin is a target to visualise angiogenesis [6]. Research into different labelled antibodies to the ED-B domain of fibronectin is in progress. "Radioiodinated anti-ED-B antibody fragments" are still under study [6], as are "anti-ED-B-single-chain fragment scFvL-19", which selectively accumulates in the tumour vasculature in a murine model [52].

VEGF is a potent cytokine that has a central role in angiogenesis during embryogenesis, neovascularisation and tumorigenesis [6]. VEGF binds to specific receptors at the cell membrane, VEGFR-1, which triggers the angiogenic response. Both VEGF and its receptor VEGFr are overexpressed in many types of solid tumours [10]. The first studies to visualise VEGF with PET were performed with antibodies, ([^{124}I]humanised anti-VEGF antibody (HuMV833)), but the results revealed low sensitivity and specificity [6, 52]. The most promising was VG76e, a monoclonal antibody labelled with ^{125}I, ^{153}S or ^{99m}Tc that binds VEGF [6].

Endostatin is an inhibitor of angiogenesis that induces apoptosis in endothelial cells [6]. In animal models for breast cancer an accumulation of [^{99m}Tc]ethylenedicysteine-endostatin ([^{99m}Tc]EC-endostatin) has been found in the endothelial tissue of the tumour [6].

Metastasis

During the growth of most tumours, the primary tumour releases cells capable of invading the vascular system and enabling distant tissues to form new tumoral foci [2, 64]. It is a complex process and some details of the regulation of the process still need to be clarified. Recently, chemokine receptors have been found to play an important role in the regulation of metastasis. The CXCR4 chemokine receptor is overexpessed in a variety of tumours. Ligands such as radiolabelled cyclic pentapeptide antagonist can quantify the expression of this receptor in animal models, but as yet there are no conclusive studies [52].

Gene expression studies

The analysis of gene expression is performed *in vitro*, in cell extracts or tissue samples. However, the development of reporter probes to specific genes designed for use in molecular imaging has allowed studies of gene expression *in vivo* of endogenous and therapeutic genes. These techniques are used mainly to validate gene therapy studies and for comparison with the levels of endogenous genes [65]. The visualisation of gene expression of endogenous genes using reporter genes and imaging techniques in live animals includes an increasing number of genes whose expression is altered in cancer [54]. These studies are carried out to determine localisation, levels and kinetic variation of gene expression *in vivo* [21].

There are three systems of reporter genes described depending on the gene product to which they correspond: an enzyme (the herpes simplex virus type 1 thymidine kinase (HSV54-tk), a receptor (the human dopamine 2 receptor (hD2R) and the human somatostatin receptor subtype 2 (hSSTR2)) or a transporter (the human sodium iodide symporter (hNIS) and norepinephrine transporter (hNET)) [66]. The better studied system is the herpes simplex virus type 1 thymidine kinase (HSV1-tk); the first studies were carried out more than 10 years ago [67]. It is used to study endogenous and therapeutic genes [14, 21, 68, 69]. The substrates proposed for the visualisation are acycloguanosine and derivatives of uracil, which are preferentially phosphorylated by the viral thymidine kinase and very little by the endogenous enzyme [21]. Table 4 includes different examples of labelled substrates to visualise different reporter genes used in gene expression studies. In order to improve the detection of low levels of this reporter gene, a mutant of herpes simples virus type 1 thymidine kinase

Table 4 Reporter genes and substrates developed to visualise gene expression

Reporter gene	Substrate	Technique	References
HSV1 tk	[^{13}I]FIAU	SPECT	1
	[^{124}]FIAU	PET	1, 21, 69
	[^{18}F]FGCV	PET	1, 20, 23, 69
	[^{18}F]FPCV	PET	20–23, 36
	[^{18}F]FHPG	PET	1, 16, 36
	[^{18}F]FHBG	PET	1, 22, 23, 36, 42
	[^{18}F]FEAU	PET	71
	[^{18}F]FFEAU	PET	71
HSV1 – sr39tk	[^{18}F]FPCV	PET	22
	[^{18}F]FHBG	PET	1, 42
	[^{124}]FIAU	PET	1, 14
cis-p537TK-GFP	[^{124}]FIAU	PET	10, 14, 16, 36, 74
hD2R	FESP	PET	1, 10, 21, 23, 42, 69
DR2-IRES-HSV1-sr39tk	FESP	PET	10, 21, 42, 43
	[^{18}F]FPCV	PET	21,
hSSTR	[^{111}In]DTPAoctreotide	SPECT	1, 68
	[^{99m}Tc]P2045	SPECT	42

FHPG, [^{18}F]fluoro-hydroxy-propoxymethyl-guanine; *FEAU*, (1-(2'-deoxy-2'-fluoro-beta-d-arabinofuranosyl)-5-ethyluridine); *FFEAU*, (1-(2'-deoxy-2'-fluoro-beta-d-arabinofuranosyl)-5-(2-fluoroethyl uridine)

(HSV1-sr39tk) has been obtained in which the enzyme is more efficient in phosphorylating ganciclovir and increases the signal of the image [21, 23]. 8-[^{3}H]penciclovir and 8-[^{18}F]fluoropenciclovir have been proposed as substrates. Studies carried out in glioma cells have revealed a two-fold increase in the accumulation of the phosphorylated product when compared with HSV1-tk wild type. HSV1-tk and the mutant HSV1-rs39tk are the systems more commonly used [70].

Many studies have been done to study transcriptional activation of p53-dependent genes considering that it appears to be mutated in almost 50% of human cancer [13]. Luker et al. have studied protein interactions, developing a reporter gene by fusing the mutant HSV1-sr39tk linked to green fluorescent protein (GFP), allowing the visualisation of the interaction of the p53 gene and the large T antigen of simian virus 40 using 8-[^{18}F]fluoroganciclovir (FGCV), 5'-iodo-2'-fluoro-2'-deoxy-1-ß-D-arabinofuranosyl-5'-iodouracil (FIAU) and fluoro-hydroxymethylbutyl-guanine (FHBG) as substrates [22, 23].

Another approach uses bicistronic constructs containing the endogenous gene of interest, an internal ribosomal entry site (IRES) and a reporter gene to visualise *in vivo* the expression of endogenous genes [21]. A method has been developed to monitor the transcriptional activity of endogenous genes [14] using a bicistronic construct with an IRES, cis-p52/TKGFP and 124FIAU PET to measure p53 transcriptional activity in tumours and transcriptional activation of genes dependent on p53. These studies demonstrated that induction of transcriptional activation of p53 correlates with genes dependent on p53 such as p21. The PET image was sensitive enough to visualise transcriptional regulation of the p53 signal transduction pathway. The viability of this system, Cis-p527TKGFP, to visualise expression of endogenous genes in a non-invasive manner has been confirmed by several authors [10, 16, 36, 69].

Recently several studies have approached the use of hypoxia inducible transcription factor 1 (HIF-1α), which mediates cellular response to hypoxia, by binding to a response element (HRE) present in different genes such as VEGF and other genes involved in stress responses. A cis-HRE/TKGFP vector has been developed to visualise HIF-1α. The first results indicate that this system could be an important tool to measure the timing of hypoxia during tumour evolution [71].

Gene expression studies have also been proposed using the endogenous gene hD2R, which together with hSSTR2 forms the second generation of reporter genes [32]. hD2R has been visualised with good results with the ligand [^{18}F] fluoro-ethyl-spiperone (FESP) for PET [1, 10, 36, 68]. Endogenous gene expression of the receptor D2R has been studied using the bicistronic vector DR2-IRES-HSV54-sr39tk and the probes FDG, FESP and 8-[^{18}F]fluoropenciclovir (FPCV). The results indicated that the signal FPCV, as a result of the expression of the SHV1-sr39tk gene, could be used to measure the level of expression of D2R [21].

hSSTR has been proposed as a potential marker for studies in humans because its expression is largely limited to tumours. Regulation of gene expression that codifies for this receptor has been visualised by PET using a protein labelled with ^{111}In, the analogue to somatostatin (octreotide), [^{111}In]DTPA-octreotide [1, 68], and [^{99m}Tc]P2045 for SPECT [42]. The third group of reporter genes are the transporters, mainly the Na/I symporter (NIS) and the norepinephrine transporter (hNET), used mainly for gene therapy studies [72].

Imaging of transcriptional regulation of endogenous genes provides information on the many cellular processes

associated with cancer [73], allowing the determination of gene profiles of different tumours [10, 74]. This helps to improve the diagnosis of different types of tumours such as breast cancer [36, 43].

Conclusions

Cancer research is a very dynamic area that provides important information about the different biological events involved in the neoplastic disease and allows advancement in the knowledge of carcinogenesis [47]. However, to finally reach the "goal" of personalised treatments [41] there are still many questions to answer [75].

In order to develop *in vivo* techniques, access to a microSPECT and/or microPET is necessary. This will allow improvement of image quality and reduction in acquisition times. The implementation of experiments with microSPECT and microPET will facilitate the development of new imaging agents for cancer research, like multimodal microPET/CT agents for use in small animals [36].

The success of molecular imaging studies depends greatly on the availability of molecular probes [52]. Therefore it is necessary to develop new probes with increased sensitivity and specificity and low toxicity [76] that can improve the imaging of the biological events under study and visualise molecules or other biological processes that still have not been studied for techniques involved in the diagnosis and staging of cancer. The development and production of molecular probes could be a long process and is necessary to validate the sensitivity, specificity and security for clinical use. As an example, for FDG this process lasted almost 20 years [41]. As a consequence of such difficulties in the production of probes labelled with ^{11}C, this radionuclide has been used very little in comparison with ^{18}F. However, considering the diversity of biological molecules, it is necessary to develop new probes labelled with ^{11}C [44].

Concerning cell proliferation it is necessary to perform additional studies to confirm the relationship between incorporation of FLT and FDG and cell growth [6]. To visualise apoptosis, it has been suggested to use sulphamides as imaging agents [52] and develop methods for more precise measurement [10]. Clearly, the hypoxic imaging technology is a promising tumoral marker, however additional studies are necessary to correlate the incorporation of the radiotracer to a specific region of the tumour, to localise the hypoxic cells and to validate its potential clinical use [6]. It is also necessary to study new molecular markers at the preclinical level to study oxygenation of the tumour, which could be useful in imaging techniques [33].

There is also a need for the development of new molecular probes to study genes involved in metastasis such as the Twist-AKT2 1 gene [25, 65]. Also, new studies are required to study other aspects related to carcinogenesis like those involving DNA repair and epigenetic effects [11, 77] or expression of tumour antigens (TAA).

An important area in the diagnosis and therapy of cancer is covered by translational research, a bidirectional process from the bench to the clinic, but also from the clinic back to the bench [78]. An editorial in Nature last year [79] clearly described the concept of translational research. In agreement with this concept, it would be very useful to have access to a synergistic platform among basic researchers and oncologists to share the latest knowledge on the molecular mechanisms of cancer that could provide the clinic with new tools for personalised diagnosis and treatment, and also have the participation of the pharmaceutical industry.

There are several techniques of molecular imaging in oncology with a high probability of being translated to the clinic [80–82], but few advances have been made due to different factors such as those related to the technology itself, financial, legal, etc. [38]. This limits the application of these techniques in clinical practice.

The role that *in vitro* techniques play in cancer research will not be replaced by *in vivo* techniques. However it is necessary to implement animal experimentation more in order to advance translational research, even though this process is taking longer than it should, for many reasons including those related to new legal regulations, which are often difficult to solve [40].

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