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## REVIEW

# Filling the gap between chemical carcinogenesis and the hallmarks of cancer: A temporal perspective

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## Abstract

**Background:** Cancer is believed to arise through the perturbation of pathways and the order of pathway perturbation events can enhance understanding and evaluation of carcinogenicity. This order has not been examined so far, and this study aimed to fill this gap by attempting to gather evidence on the potential temporal sequence of events in carcinogenesis.

**Design:** The methodology followed was to discuss first the temporal sequence of hallmarks of cancer from the point of view of pathological specimens of cancer (essentially branched mutations) and then to consider the hallmarks of cancer that one well-known carcinogen, benzo(a)pyrene, can modify.

**Results:** Even though the sequential order of driving genetic alterations can vary between and within tumours, the main cancer pathways affected are almost ubiquitous and follow a generally common sequence: resisting cell death, insensitivity to antigrowth signals, sustained proliferation, deregulated energetics, replicative immortality and activation of invasion and metastasis. The first 3 hallmarks can be regarded as almost simultaneous while angiogenesis and avoiding immune destruction are perhaps the only hallmarks with a varying position in the above sequence.

**Conclusions:** Our review of hallmarks of cancer and their temporal sequence, based on mutational spectra in biopsies from different cancer sites, allowed us to propose a hypothetical temporal sequence of the hallmarks. This sequence can add molecular support to the evaluation of an agent as a carcinogen as it can be used as a conceptual framework for organising and evaluating the strength of existing evidence.

## KEYWORDS

“meet-in-the-middle” approach, benzo(a)pyrene, cancer, carcinogenesis, hallmarks of cancer, temporal sequence

## 1 | INTRODUCTION: PATHWAYS TO CANCER

Cancer is believed to arise through the perturbation of pathways or networks, with a minimum number of steps required for clinical cancer initiation. Pathway/network

perturbation events can provide a selective advantage either directly (mutagenesis) or indirectly (selection of advantageous mutations). The order of pathway perturbation events following exposures to environmental stressors can enhance understanding and evaluation of carcinogenicity, but has not been examined so far, mainly for practical limitations in the study of precancerous events in humans. In addition, there has been a disconnection between mechanistic

Robinson and Vineis equally contributed to this study.

**TABLE 1** The hallmarks of cancer (from ref. 1)

Hallmarks
Sustaining proliferative signalling
Evading growth suppressors
Resisting cell death
Enabling replicative immortality
Inducing angiogenesis
Activating invasion and metastasis
Reprogramming of energy metabolism
Evading immune destruction
Enabling events
Genome instability and mutation
Inflammation

research culminating in the paradigm of the “hallmarks of cancer”<sup>1</sup> (Table 1) and research on the causes of cancer, including viral- and chemical-induced carcinogenesis. These are 2 worlds that barely communicate.

This study aimed to fill this gap by attempting to gather evidence on the potential temporal sequence of events in carcinogenesis, while acknowledging that cancer progression may not be a fixed pathway but one that demonstrates substantial variability. A suggested temporal order of the hallmarks would add molecular support to the evaluation of an agent as a carcinogen as it can be used as a conceptual framework for organizing and evaluating the strength of existing evidence concerning steps necessary for progressing from molecular initiating events to an adverse outcome. Furthermore, in the attempt to integrate mechanistic data in carcinogen evaluations, based on a “meet-in-the-middle” (MITM) approach,<sup>2</sup> knowledge of the temporal sequence of events in carcinogenesis would be precious. This is because cancer, just as any disease, develops over time, and understanding its aetiology has to include temporal clues. The MITM concept is at the basis of current research in molecular epidemiology and implies the identification of molecular changes that are intermediate between previous exposures and later outcomes in longitudinal settings. The reconstruction of such a temporal order would enable establishing the relationship between the *middle-to-*

*outcome* nature of the hallmarks of the cancer phenotype and the *bottom-to-middle* approaches of the key characteristics of carcinogens<sup>3</sup> (Figure 1), and whether these (so far separate) events converge towards a suggested temporal sequence. In other words, elucidating the order of cancer hallmarks would allow a more detailed investigation into whether there are hallmarks (related to cancer phenotypes) that *overlap* with key characteristics of carcinogens (related to carcinogen exposure), thus demonstrating potential “meet-in-the-middle” networks. This proof-of-principle can be applied to mechanistic evidence on potential carcinogens, thus strengthening carcinogen evaluations and causal reasoning.

This methodological strategy is supported by a particular concept of causation, defined as information transmission. The challenge of correctly understanding the aetiology of cancer rests on the possibility of identifying key moments of its temporal development. A molecular approach, through the “meet-in-the-middle” methodology, can identify biomarkers that *mark* these salient moments. In the *process* of cancer development, causality is the transmission of information, which we intercept at the molecular level, and that we contextualize with mechanistic data. Illari and Russo,<sup>4</sup> in this sense, conceive of (cancer) mechanisms as the channels in which information flows. Vineis et al<sup>5</sup> further explain that this concept makes causality an important part of the scientists’ activity to *interpret* results and to *combine* different lines of research—here the “hallmarks of cancer” and the molecular approaches to carcinogenesis.

## 2 | THE TEMPORAL ORDER OF GENETIC ALTERATIONS IN CARCINOGENESIS

This review discusses first the temporal sequence of hallmarks of cancer from the point of view of pathological specimens of cancer (essentially branched mutations) and then considers the hallmarks of cancer that one well-known carcinogen, benzo(a)pyrene (BaP), can modify.

The sequential order of genetic alterations has been shown to vary between and within tumours. The following is an overview of studies which have examined the genomic alterations in the branched evolution of different tumours and/or different sections within a single tumour.

The premise of these studies is that if a gene is found mutated with a high frequency in multiple tumours from many different patients, or in many samples from the same tumour, it is less likely to be a passenger mutation and more likely to provide the cell with a selective advantage, permitting it to expand and eventually dominate the cell population (Box 1). Also, ubiquitous genetic alterations between several samples from the same tumour are more



**FIGURE 1** The components of the meet-in-the-middle approach: bottom to middle refers to the action of carcinogenic agents, and middle to top refers to the appearance of the cancer phenotype according to hallmarks of cancer

likely to have occurred early in the evolution of the cancer, whereas later alterations, present only in some tumour sections, most likely contributed to later tumour branching.

In our understanding of the causal process of cancer, gene mutations could be salient events in the whole process of cancer development. Their temporal occurrence and their relation to the hallmarks of cancer contribute to reconstruct the information flow from cancer onset to its development.

The evidence in the reviewed studies is explored in an attempt to place the hallmarks of cancer in a temporal order of appearance. According to the model we refer to (Table 1), there are 8 *hallmarks of cancer* (sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism and evading immune destruction) and 2 *enabling events* (genome instability and mutation and inflammation).

## 2.1 | Colorectal cancer

Colorectal cancer is the most frequently examined tumour with respect to its genetic alterations and branched evolution.

In 95 colorectal cancer samples, using the Hidden Conjunctive Bayesian Network (H-CNB) model, researchers identified *APC* mutations as initiating events (accumulation rate (AR) 0.39 per year—consistent for an early driving role), followed by *KRAS* (AR = 0.12 per year), *PIK3CA* (AR = 0.009 per year) and other mutations such as *EVC2*, *FBXW7*, *EPHA3* and *TCF7L2*.<sup>6</sup> In parallel, *TP53* (AR = 0.06 per year) mutations could be placed either before or after *APC* and *KRAS* mutations as their presence was independent of the presence of *APC* and *KRAS* mutations.<sup>6</sup>

The model places key signalling pathways in the following order: small GTPase pathway, apoptosis/Wnt/Notch signalling and homophilic cell adhesion. These alterations are often found before alterations in *KRAS* and TGF- $\beta$  signalling pathways and before alterations in G1/S phase control. DNA damage control and JNK are altered at later stages, probably through *TP53* mutations. Integrin signalling and invasion are not as common, suggesting roles at later stages of colon carcinogenesis.

Translating the pathways into hallmarks, the evidence above places evasion of growth suppressors, sustaining proliferative signalling and resisting cell death prior to avoiding immune destruction and activating invasion and metastasis. Angiogenesis occurs simultaneously with initiation of invasion, whereas immortality occurs in parallel with the hallmarks prior to invasion and metastasis.

The early presence of the *APC* and *KRAS* mutations, as well as their high mutation accumulation rates (ARs: 0.39

and 0.12 per year<sup>6</sup>), is consistent with an early driving role.

These findings are in agreement with the Fearon and Vogelstein model of colorectal carcinogenesis<sup>7</sup> where *APC*, a tumour suppressor gene, is firstly inactivated, allowing the normal epithelium to become hyperplastic and eventually form an early adenoma. Activation of *KRAS* then contributes to sustained proliferation and the formation of an intermediate adenoma, whereas further loss of tumour suppressors, such as *Smad4*, contributes to the formation of a late adenoma. The loss of p-53 then allows resistance to cell death and immortality. Additional genetic alterations, such as the accumulated loss of suppressor genes on additional chromosomes, correlate with the ability of the carcinomas to metastasize and cause death. However, Fearon and Vogelstein emphasize the importance of the accumulation of these changes, rather than their temporal order which may vary.

Rosenberg et al<sup>8</sup> also identified mutations in the *APC* and *KRAS* genes as an early event in rodent colon cancer models. Even in early, premalignant lesions, increased proliferative activity, growth factor signalling and *KRAS* mutations were evident.

In addition, Wood et al<sup>9</sup> in their analysis of 11 colorectal tumours identified *APC*, *KRAS* and *TP53* as the most frequent driving mutations, followed by mutations in *PIK3CA*, *FBXW7*, *CSMD3*, *TNN*, *NAV3*, *SMAD4* and many more. All these genes have passenger probability scores of <.0001.

Independently, Beerenwinkel et al<sup>10</sup> also identified *APC*, *TP53* and *KRAS* as the most often mutated genes in their analysis of 78 candidate cancer genes in 35 tumour (colon adenoma and carcinoma) samples. Therefore, evasion of growth suppressors, resisting cell death and sustained proliferation are again evidenced as the first cancer hallmarks to appear.

With respect to the temporal order of angiogenesis, studies corroborate its simultaneous occurrence with the initiation of invasion. Evidence from Takahashi et al<sup>11</sup> places the angiogenic switch between mucosal and submucosal invasive cancer. Hanahan and Folkman<sup>12</sup> also place angiogenesis prior to solid tumour formation but following hyperproliferation. In addition, Zhang et al<sup>13</sup> place angiogenesis in early premalignant stages of tumour development. Furthermore, in benign colorectal adenomas, vascular endothelial growth factor (VEGF) protein and RNA levels exceed those of normal colonic mucosa.<sup>14,15</sup> Lastly, the presence of VEGF A and B at the adenoma stage<sup>16</sup> further supports the order of the angiogenic switch sometime after hyperproliferation and prior to invasion.

Lastly, even though *APC*, *TP53* and *KRAS* mutations were identified at a high frequency in different sections from the primary tumour of a patient with colorectal

**BOX 1**

Technical definitions:

- Mutation accumulation rate (AR): Estimated yearly accumulation rate for a particular mutation. The higher the accumulation rate, the higher the frequency of occurrence of the mutation in different tumour samples.
- Driver mutations: Mutations involved causally in the neoplastic process, conferring a selective advantage, thus being positively selected for during tumorigenesis.
- Passenger mutations: Mutations that provide no positive or negative selective advantage to the tumour but are retained by chance during repeated rounds of cell division and clonal expansion.
- Passenger mutation rates: Estimated through the quantification of synonymous (silent) missense mutations, because such mutations are expected to be biologically inert and can therefore exert no positive or negative selective advantage.
- Passenger Probability Scores: These gene-specific scores are based on a likelihood ratio test (LRT) for the null hypothesis that, for the gene under consideration, the mutation rates are the same as the passenger mutation rates. Small probability scores support rejection of the null hypothesis and acceptance that mutation rates are in fact higher than the passenger mutation rates. Therefore, small passenger probability scores support a “driver” role for mutations in a particular gene.

cancer,<sup>17</sup> only one section from this tumour contained *PIK3CA* mutations, at a low frequency. Interestingly, the metastatic tumour from the same patient harboured *PIK3CA* mutations at a high frequency. This evidence supports the temporal order of replicative immortality just prior to invasion and metastasis.

## 2.2 | Pancreatic cancer

Using the H-CNB to model 90 cases of pancreatic cancer, mutations in *KRAS* (AR > 100 per year, prevalence = 100%) appear to initialize progression followed by *TP53* (AR = 0.34 per year), *CDKN2A* (AR = 0.013 per year) and *MLL3* (AR = 0.00066). *SMAD4* is also mutated independently (AR = 0.015 per year).<sup>6</sup>

Extrapolating to pathway level, apoptosis, G1/S transition, Hedgehog and TGF- $\beta$  signalling pathways was ubiquitous, indicating their alteration at the earliest stages of

pancreatic carcinogenesis. Then, alterations in small GTPase-dependent signalling and *KRAS* signalling arise independently, followed by alterations in DNA damage control, JNK and Wnt/Notch signalling. These alterations also contribute to replicative immortality. Integrin signalling is altered at late stages after homophilic cell adhesion, further contributing to senescence. Lastly, invasion pathways were found unaltered in these samples, suggesting roles in even later stages of carcinogenesis.

This model indicates that in pancreatic cancer, the hallmarks of evading growth suppressors, sustaining proliferative signalling, resisting cell death and inducing angiogenesis, are followed by deregulating cellular energetics, which in turn precedes evading immune destruction, and activating invasion and metastasis. The model does not allow temporal placing of immortality.

Interestingly, quantitative analysis of the timing of the genetic evolution of pancreatic cancer, based on cell proliferation rates of normal pancreatic tissue and pancreatic metastatic tumours, suggested that at least a decade passes between the initial driving mutation and the birth of the parental, nonmetastatic founder cell and at least 5 more years are needed for the acquisition of metastatic ability.<sup>18</sup> However, these numbers are mathematical estimates based on a small number of tumour samples; therefore, the evidence for the timing is weak.

## 2.3 | Primary glioblastoma

In 78 primary glioblastoma tumours, *TP53* (AR = 0.015 per year), *PTEN* (AR = 0.012 per year), *EGFR* (AR = 0.0026 per year), *NF1* (AR = 0.0043 per year), *PI3CA* (AR = 0.033 per year), *IDH1* (AR = 0.0087 per year), *PIK3R1* (AR = 0.0037) and *RBI* (AR = 0.011) were identified as the most commonly mutated genes.<sup>6</sup>

The H-CNB model identified *TP53* as the first mutation, with *NF1*, *PTEN* and *EGFR* being mutated in parallel. Mutations then proceed in the following order: *PIK3CA*, *PIK3R1* and *RBI*. However, the low accumulation rates of these individual mutations indicate a low probability for a specific gene alteration in primary glioblastomas.

This mutation order highlights apoptosis and small GTPase pathways as the first and most commonly affected pathways. G1/S phase transition, Wnt/Notch signalling and *KRAS* signalling were also mutated early. Alterations in these pathways are followed by alterations in DNA damage control, JNK signalling, homophilic cell adhesion and integrin signalling, the latter 2 occurring independently of the former 2 pathways. No alterations in the invasion pathway were identified.

Therefore, translating the pathways into hallmarks, in primary glioblastomas, sustaining proliferative signalling and resisting cell death are the first hallmarks identified,

followed by deregulated cellular energetics, evasion of growth suppressors and induction of angiogenesis. *TP53* alterations are also likely to initiate the process of replicative immortality which is completed at later stages with *PIK3CA* and *RB1* mutations. Invasion and metastasis are the last hallmark to occur, after avoiding immune destruction.

Independently, Parsons et al<sup>19</sup> investigated the frequency of mutations in 105 samples of glioblastoma tumours. They found *CDKN2A*, *TP53*, *EGFR*, *PTEN*, *NF1*, *CDK4*, *RB1*, *IDH1*, *PIK3CA* and *PIK3R1* as the most frequently altered genes, in this order. With the exception of *PIK3CA* and *PIK3R1*, all other gene alterations have a passenger probability of <.01, indicating their role as driving mutations.

Based on these genes' functions and assuming that the most prevalent/driving alterations are necessary for initiation of carcinogenesis, whereas less frequent alterations have later roles in the carcinogenesis cascade, then in glioblastoma tumours, cancer hallmarks are expected to appear in the following order: evasion of cell death, insensitivity to antigrowth signals, sustained cell proliferation, deregulated energetics (Warburg effect), immortality and invasion and metastasis. Unfortunately, this list does not allow estimation of the temporal position of angiogenesis and evasion of immune destruction in glioblastoma tumours.

## 2.4 | Renal carcinoma

Gerlinger et al<sup>20</sup> studied intratumour branched evolution in 2 renal cell carcinomas by multiregion sequencing and identified that *VHL*, *SETD2*, *PTEN* and *KDM5C* underwent multiple, distinct and spatially separated inactivating mutations.

In the first patient, *VHL* was identified as the first, driving mutation, followed by *SETD2* mutations which break the phylogenetic tree in 2 branches. In one branch (core biopsy samples), heterogeneity is further propagated by *KDM5C* and *mTOR* mutations. In the second branch (metastases samples), *SETD2* mutations are also followed by *KDM5C* mutations.

*VHL*, the gene identified as firstly mutated, is involved in cell division and formation of new blood vessels, among other functions, identifying cell proliferation and perhaps initiation of angiogenesis as early, widespread events in renal carcinogenesis. The *SETD2* gene normally trimethylates histone 3 lysine 36 at sites of active transcription. Its mutation results in silencing of transcription instead of an active chromatin conformation. This leads to altered nucleosome dynamics and DNA replication stress, as well as to failure in loading lens epithelium-derived growth factor and the Rad51 homologous recombination repair factor at DNA breaks.<sup>21</sup> Therefore, growth factor evasion follows sustaining proliferative signalling. *KDM5C*, which is mutated

downstream, is involved in regulation of transcription through transcriptional repression, which most likely contributes to maintaining genetic instability. Lastly, *mTOR* is a target for cycle arrest, and immunosuppressive effects, and when mutated, it is involved in deregulated cellular energetics, evasion of growth suppressors, genetic instability and replicative immortality,<sup>22</sup> suggesting that immune system evasion and replicative immortality are later events.

In the second patient, *VHL* and *PBRM1* were mutated in all tumour specimens followed by *SETD2* mutations which break the phylogenetic tree in 2 branches. In one branch, the metastatic branch, *SETD2* mutations are accompanied by *P53* mutations. In the other branch, the core tumour branch, *PTEN* mutations lead to further branching.

*PBRM1* is necessary for ligand-dependent transcriptional activation and is involved in chromatin organization, whereas *PTEN* is a tumour suppressor enzyme which regulates cell division, apoptosis, cell movement, adhesion and angiogenesis. *P53* is one of the most well-established tumour suppressors associated with all cancer hallmarks, namely increased cancer metabolism, angiogenesis, genetic instability, immune evasion, resistance to cell death, replicative immortality, sustained proliferative signalling, invasion and metastasis.<sup>23</sup> This branching further confirms that sustaining proliferative signalling, inducing angiogenesis and transcriptional activation are widespread in tumours and occur early. They are followed by evasion of growth suppressors through repression of antigrowth signals, via chromatin inactivation (*SETD2* mutations) and antigrowth factor inactivation (*PTEN* and *P53* mutations). Lastly, this branching suggests that other hallmarks such as replicative immortality, invasion and metastasis, as mediated by *P53*, follow at later stages of renal carcinogenesis.

## 2.5 | Melanoma

Multiregion sequencing in 41 multiple melanoma biopsies from 8 individual tumours<sup>24</sup> found that the 3 melanoma driver genes, namely *BRAF*, *NRAS* and *NF1*, all key components of the MAPK pathway, were ubiquitously mutated in a mutually exclusive pattern. The ubiquitous expression of these genes is consistent with an early role of this pathway (involved in sustaining proliferative signalling and evasion of growth suppressors) in melanoma formation. On the contrary, mutations in the PI3K pathway were heterogeneous indicating that such mutations occur later in metastatic melanoma evolution. As the PI3K is important for many cell activities, including cell growth and division (proliferation), movement (migration) of cells and cell survival, its appearance later in carcinogenesis suggests later appearance of the hallmarks of resistance to cell death, and activation of invasion and metastasis. *IDH1*, which has an essential role in glucose metabolism, is also mutated

heterogeneously in some melanoma tumours, suggesting later appearance of the Warburg effect.

## 2.6 | Breast carcinoma

In an analysis of 11 breast cancer tumours, Wood et al<sup>9</sup> identified *TP53* and *PIK3CA*, as the most frequent, driving mutations. The 2 genes have passenger probability scores of <.0001, indicating their important driving roles in carcinogenesis. Assuming that the most prevalent/driving alterations are necessary for initiation of carcinogenesis, the high mutation frequency of these genes highlights the early appearance of the resisting cell death, evading growth suppressors and sustaining proliferative signalling cancer hallmarks.

## 2.7 | Combined evidence on different tumour types

Looking at colon, pancreatic and glioblastoma tumours together, the H-CNB model<sup>6</sup> identified the following order of pathway alterations as the most likely: apoptosis, TGF- $\beta$  signalling, small GTPase-dependent signalling (other than *KRAS*), Wnt/Notch signalling, control of G1/S phase transition, *KRAS* signalling, Hedgehog signalling, DNA damage control, JNK, homophilic cell adhesion, integrin signalling and invasion.

Furthermore, Raica et al<sup>25</sup> reviewed angiogenesis in premalignant lesions and concluded that tumour angiogenesis is not necessarily a characteristic of invasive tumour, but may occur prior to malignancy, as defined by invasion and metastasis. In their review, they gather evidence that microvessel density (MVD) was significantly increased in a relatively large spectrum of premalignant squamous cell lesions, such as in the oral mucosa, skin, uterine cervix, vulva and anal canal. Interestingly, for a number of these lesions, MVD was found to correlate with the major pro-angiogenic factor, namely VEGF.

Premalignant lesions of glandular epithelia, including gastric metaplasia and dysplasia, atypical adenoma of the colon, atypical hyperplasia and carcinoma in situ of the breast also exhibited VEGF overexpression.

Based on the above evidence, the following order of hallmarks emerges: resistance to cell death, insensitivity to antigrowth signals, sustaining proliferative signalling, deregulated cellular energetics, inducing angiogenesis, avoiding immune destruction, enabling replicative immortality and invasion and metastasis.

## 2.8 | Comments on temporal sequence of hallmarks

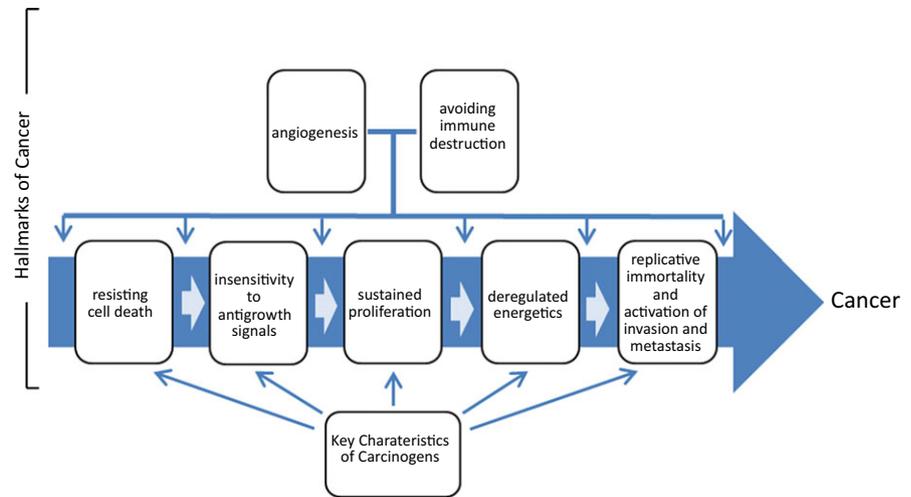
From the studies we have reviewed, it occurs that even though tumour cells can accumulate many mutations as

they evolve, not all of these mutations are driving mutations (ie play a causal role). Some mutations are just a by-product of the increased genetic instability which characterizes the process of tumorigenesis.<sup>26</sup> In the causal framework we adopt, this means that mutations can provide different cues for the temporal reconstruction of cancer development. In other words, mutations may mark the cancer process at different salient points.

Studies have shown that a typical tumour contains 2-8 “driver gene” mutations,<sup>26,27</sup> but which specific genes will be mutated in different tumours might vary. In addition, cells from different sections of the same tumour tend to have a diverse mutational landscape, suggesting that different mutations may converge towards the same phenotypic result. This is because the intracellular pathways often deregulated in cancer (pathways that drive carcinogenesis) incorporate many genes which operate as an *information transfer cascade*.<sup>28</sup> Mutations in any one of the genes can have a similar effect, that is stopping the cascade and the transfer of information. This view is supported by the observation that multiple hits in different genes of the same pathway in individual tumours are less frequent than expected<sup>29</sup> and in different tumours, different members of each pathway may be affected in a mutually exclusive manner.<sup>19</sup> Furthermore, different cancer specimens of the same tumour type carry their individual cancer driver gene mutational signatures, which seem to be mutually exclusive as the number of common mutant cancer driver genes between tumours is limited.<sup>30</sup>

Even though the sequential order of driving genetic alterations can vary between and within tumours, and even though in different tumours different members of each pathway may be affected in a mutually exclusive manner, the main cancer pathways affected are almost ubiquitous and follow a generally common sequence. Looking at the combined evidence from different tumour types (Figure S1), these cancer pathways and their sequential alterations enable the suggestion of a temporal sequence for the appearance of cancer hallmarks: resisting cell death, insensitivity to antigrowth signals, sustained proliferation, deregulated energetics, replicative immortality and activation of invasion and metastasis. The first 3 hallmarks can be regarded as almost simultaneous as their exact order is switched in different investigations. Angiogenesis and avoiding immune destruction are perhaps the only hallmarks with a varying position in the above sequence, but they are most often evidenced to follow deregulated energetics and to precede replicative immortality, invasion and metastasis.

In conclusion, the evidence on tumour biopsies proposes a sequence for 5 of the hallmarks, with the others potentially residing in different places along the path, as indicated in Figures 2 and S1.



**FIGURE 2** Tentative reconstruction of a sequence of hallmarks based on mutations in biopsies (see text). It should be noted that the first 3 steps can occur in varied order. Also, we do not consider in the figure the fact that both mutations and clone selection operate in carcinogenesis

### 3 | BENZO[A]PYRENE AND THE HALLMARKS OF CANCER

We now consider the events associated with the carcinogenicity of a well-known carcinogen, using the hallmark of cancer paradigm. We refer for simplicity (and comparability with the paragraph above) to the original Hanahan and Weiberg<sup>1</sup> paper rather than to its adaptation to “key characteristics of carcinogens” by Smith et al<sup>3</sup> (Table 2).

Benzo[a]pyrene (*BaP*) is a ubiquitous contaminant, belonging to the large group of organic compounds with 2 or more fused aromatic (benzene) rings, namely polycyclic aromatic hydrocarbons (PAHs).<sup>1,31,32</sup> PAHs and *BaP* formed during incomplete combustion, and their major sources include tobacco smoke, residential and commercial heating with wood or coal, motor-vehicle exhaust and industrial emissions.<sup>31</sup> Occupational exposures occur in aluminium production, roofing and paving involving coal-tar pitch, coal liquefaction, coal-tar distillation, wood impregnation, chimney sweeping and power plants<sup>33</sup>. While no epidemiological studies on *BaP* alone are available, *BaP* produced tumours in multiple organs and tissues in all animal species tested following exposures by many different routes.<sup>31</sup> Mechanistic evidences from in vitro and in vivo studies, including human exposures, showed that *BaP* is clearly genotoxic following its metabolism into highly reactive species that form DNA adducts leading to sister chromatid exchange, chromosomal aberrations, micronuclei or DNA damage.<sup>31</sup> Based on these multiple experimental evidences, *BaP* may be considered a well-established model carcinogen. Thus, we decided to review the literature available on *BaP* and its capacity to affect the different hallmarks of cancer.

#### 3.1 | Hallmark 1: Sustaining proliferative signalling

*BaP* is a very well-known chemical able to immortalize human cells, such as breast epithelial cells.<sup>34</sup> One of the

earliest mechanistic evidence of *BaP* effects on cell proliferation was its ability to form DNA adducts, and recurrent mutations were observed in the genes of the *RAS* superfamily both in humans and in mice.<sup>35-37</sup> Beyond the mutagenic effects on genes associated with cell proliferation, Kometani et al<sup>38</sup> showed that *BaP* was able to promote proliferation of human lung cancer cells after 24 weeks of exposure by activating the EGFR pathway through induction of EGFR ligands, amphiregulin and epiregulin. Moreover, *BaP* may promote cell proliferation through the activation of nuclear receptor signalling pathways, such as aryl hydrocarbon receptor (AhR) and oestrogen receptor (ER) in humans, mice and other nonmammalian organisms.<sup>39-42</sup> In conclusion, *BaP* is able to induce cell proliferation through both genotoxic and nongenotoxic receptor-induced mechanisms in humans and various experimental models.

#### 3.2 | Hallmark 2: Evading growth suppressors

*BaP* exposure allows cells to evade G1 arrest and induces cell abnormal proliferation.<sup>43</sup> Most studies reported an activation of the ERK pathway in different cell types.<sup>43-45</sup> Interestingly, a short exposure of 24 hours was sufficient to induce a dose-related activation of MAPK in normal human embryo lung diploid fibroblasts,<sup>43</sup> and apparently, cellular response to *BaP* exposure was also dependent on the growth kinetics within a target cell population,<sup>44</sup> suggesting different susceptibility based on cell state and differentiation.

#### 3.3 | Hallmark 3: Resisting cell death

A recent exomewide mutation profile on immortal human mammary epithelial cells exposed to *BaP* showed that genes involved in various biological processes, including regulation of cell death, harbour mutations predicted to

**TABLE 2** Key characteristics of carcinogens

Is electrophilic or can be metabolically activated
Parent compound or metabolite with an electrophilic structure (eg, epoxide or quinone), formation of DNA and protein adducts
Is genotoxic
DNA damage (DNA-strand breaks, DNA-protein crosslinks or unscheduled DNA synthesis), intercalation, gene mutations, cytogenetic changes (eg, chromosome aberrations or micronuclei)
Alters DNA repair or causes genomic instability
Alterations of DNA replication or repair (eg, topoisomerase II, base-excision or double-strand break repair)
Induces epigenetic alterations
DNA methylation, histone modification, microRNA expression
Induces oxidative stress
Oxygen radicals, oxidative stress, oxidative damage to macromolecules (eg, DNA or lipids)
Induces chronic inflammation
Increased white blood cells, myeloperoxidase activity, altered cytokine or chemokine production
Is immunosuppressive
Decreased immunosurveillance, immune system dysfunction
Modulates receptor-mediated effects
Receptor activation or inactivation (eg, ER, PPAR and AhR) or modulation of endogenous ligands (including hormones)
Causes immortalization
Inhibition of senescence, cell transformation
Alters cell proliferation, cell death or nutrient supply
Increased proliferation, decreased apoptosis, changes in growth factors, energetics and signalling pathways related to cellular replication or cell cycle
Any characteristic could interact with any other (such as oxidative stress, DNA damage and chronic inflammation), and a combination provides stronger evidence of a cancer mechanism than one would alone.

Sources: Smith et al<sup>3</sup>.

impact protein function.<sup>46</sup> However, other *in vitro* reports showed that exposure to BaP or its DNA-reactive metabolite anti-benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE) induces or sensitizes human cells to either receptor-mediated or mitochondrial-mediated apoptosis.<sup>47,48</sup> Similar results were obtained using a reverse phase protein array comparing mouse primary liver tumours induced by BaP to their normal adjacent tissues.<sup>49</sup> The results showed both downregulation (eg cleaved caspase 7, caspase 3) and upregulation (Bax, Bad and Bcl-xL) of some pro-apoptotic proteins in tumour tissues. In conclusion, BaP may exert both a pro-apoptotic or an anti-apoptotic effect. *In vivo* data on the temporal sequence of these effects are lacking, although it has been suggested that the carcinogenic effect of BaP or PAHs mixtures may be also mediated by chronic inflammation and cell death subsequent to the exposure.<sup>50</sup>

### 3.4 | Hallmark 4: Inducing angiogenesis

It has been recently shown that 1-month exposure to different BaP concentrations increased in a dose-dependent manner the capacity of a hepatoma cell lines,

BEL-7404, to recruit vascular endothelial cells and promote angiogenesis through increased secretion of VEGF.<sup>51</sup> Similarly, low, noncytotoxic concentration of BaP induced hypoxia-inducible factor-1 $\alpha$ , responsible for the adaptation to hypoxic conditions and the promotion of angiogenesis.<sup>52</sup> Interestingly, BaP and its metabolites may have distinct and opposite effect on VEGF expression,<sup>53</sup> suggesting that tissue-specific or genetic interindividual differences in the CYP450 expression may play a role in determining the overall effect on angiogenesis induction by BaP and PAHs in general.

### 3.5 | Hallmark 5: Enabling replicative immortality

BaP has been shown to induce efficiently immortalization of Syrian hamster normal dermal cells through its mutagenic activity causing the direct inactivation of *p53* and INK4 alterations.<sup>54,55</sup> Similarly, *p53* mutations in Hupki cells (embryonic murine fibroblast with human *p53* gene) exposed to BaP were correlated with *p53* mutations in human lung tumours, supporting the direct role of BaP in

causing smokers lung tumour *p53* mutations.<sup>56</sup> In conclusion, BaP seems to enable replicative immortality mainly through its genotoxic properties.

### 3.6 | Hallmark 6: Activating invasion and metastasis

There is evidence that BaP can promote cell migration, invasion and metastasis.<sup>57</sup> Recently, some studies provided mechanistic clues showing that BaP may contribute to lung cancer cell invasion and metastasis by upregulating pro-inflammatory chemokines (IL8, CCL-2, CCL-3) and the one of the master regulator of the epithelial-to-mesenchymal transition, Twist.<sup>58,59</sup> Moreover, BaP was able to induce cell migration in triple-negative breast cancer MDA-MB-231 cells through a lipoxygenase- and Src-dependent pathway, notably by increasing the secretion of metalloproteinase MMP-2 and MMP-9.<sup>60</sup> BaP treatment was also able to increase the metastatic potential of hepatocellular carcinoma cell lines in a mouse model, likely through an activation of both angiogenesis and NF- $\kappa$ B pathway.<sup>51</sup> In conclusion, BaP is able to promote cell migration and invasion, contributing to increase the metastatic potential of several epithelial cells in different experimental settings.

### 3.7 | Hallmark 7: Deregulating cellular energetics

Recent studies showed that BaP is able to alter the function of mitochondria, cellular organelles having a key role in cellular energetics as well as in programmed cell death. In particular, BaP (range 0-500  $\mu$ mol/L) was able to lower mtDNA content in a TK6 cell, a human lymphoblastoid cell line.<sup>61</sup> Interestingly, in the same study, indoor exposure to PAHs was associated with decreased mtDNA content in the blood.<sup>61</sup> Similarly, BaP induced mitochondrial damage in vivo in mice cervical tissues, strongly associated with increased oxidative stress.<sup>62</sup> Interestingly, a short-term exposure (24-48 hours) to BaP induced an increased expression of several components of the mitochondrial respiratory chain,<sup>63</sup> suggesting an adaptive process that could cause a mitochondria-derived increased oxidative stress to the exposed cells and tissues. In conclusion, while a more metabolism-focused research on the effects of BaP is needed, some evidence exists that it could directly impact cellular metabolism and energy production, notably through an alteration of the mitochondrial function.

### 3.8 | Hallmark 8: Avoiding immune destruction

Early studies in the 1980s showed BaP has immunotoxic effects in mice, by reducing antibody production and

inducing DNA adducts in splenic leucocytes.<sup>64,65</sup> A more recent study showed that BaP at a dose as low as 10 mg/kg b.w. (generally considered as nontoxic) was able to induce changes in thymus weight and spleen B-cell populations.<sup>66</sup>

In conclusion, while no evidence was available to test the hypothesis that BaP may affect cancer cells immune recognition, BaP is able to exert immunotoxicity, thus contributing to avoiding immune destruction by cancer cells.

### 3.9 | Enabling characteristics: Genome instability and mutation and tumour-promoting inflammation

The acquisition of the hallmarks of cancer is made possible by 2 enabling characteristics, namely genome instability (which allows accumulation of random mutations) and the inflammatory state accompanying many premalignant and malignant lesions.<sup>1</sup> As a genotoxic compound, BaP and its metabolites are a well-known mutagen for both human and experimental animals. Interestingly, a recent study involving next-generation sequencing of exomes in immortal human mammary epithelial cells showed that DNA repair genes were among the genes harbouring BaP-induced mutation with a potential functional impact, suggesting a potential impact on genome stability of the mutated cells.<sup>26</sup> Moreover, BaP increased the number of oxidatively induced clustered DNA lesions in normal primary breast-derived cells which were correlated with the number of chromosomal aberrations.<sup>67</sup> These lesions were associated with a decrease in antioxidant defence capacity and an increased ROS and DNA repair gene transcription,<sup>67</sup> suggesting that oxidative stress and DNA damage and repair response are strictly correlated following BaP exposure.

Important crosstalks between chemicals and immune system in carcinogenesis have been recently reviewed.<sup>68</sup> Interestingly, interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF- $\alpha$ ) produced by macrophages have been shown to be critical to promote malignant transformation of human bronchial epithelial cells in a bionic airway chip culture system and in an animal model (Li et al 2015). Similarly, it was observed that TNF- $\alpha$  strongly augmented the formation of stable BaP diol epoxide-DNA adducts in alveolar type II epithelial cells.<sup>69</sup> Thus, the inflammatory response to PAHs containing BaP and the immunosuppressive ability of BaP towards acquired immunity cells previously described may synergistically cooperate in the carcinogenicity of BaP aPAHs mixtures.

A recent review provided in the context of the Halifax project<sup>50</sup> described Benzo(a)pyrene (BaP) as a prototypical multidisruptor influencing sustained proliferation during carcinogenesis. Interestingly, they found that the mechanisms leading to this phenotype could not be entirely

explained by the genotoxicity of its metabolites. In particular, for PAHs mixtures, mechanisms involving chemical-receptor interaction, notably with oestrogen receptor and aryl hydrocarbon receptor, have been described.<sup>70</sup> Moreover, other than nuclear receptor activation, BaP was also shown to induce epigenetic alterations, affecting DNA methylation, histone modification and non coding RNA expression.<sup>71</sup> Finally, similarly to the conclusion based on our literature review, BaP was found associated with most hallmarks of cancer,<sup>50</sup> but there was no evidence to support a temporal sequence of events. In conclusion, BaP may exert its multipotent and multitissue carcinogenic effects by affecting all known hallmarks of cancer and by displaying multiple key characteristics of carcinogens. Even at low concentrations and as part of chemical mixtures, BaP and PAHs perturb multiple signalling pathways, including hormonal pathways and those regulating energy metabolism, depending on the status of the target cell.

#### 4 | CONCLUSIONS: SUGGESTIONS FOR “MEET-IN-THE-MIDDLE” RESEARCH

Our review of hallmarks of cancer and their temporal sequence, based on mutational spectra in biopsies from different cancer sites, allowed us to propose a hypothetical temporal sequence of the hallmarks: resisting cell death, insensitivity to antigrowth signals, sustained proliferation, deregulated energetics, replicative immortality and activation of invasion and metastasis. On the other side, it was not possible to isolate a clear sequence of the cancer hallmarks for the effects of the model carcinogen we have chosen, Benzo (a)pyrene. Also, it was not possible to find a clear overlay of hallmarks in the cancer sites we have examined and the hallmarks affected by Benzo(a)pyrene. In particular, it seems that the latter has a multiplicity of mechanisms or modes of action, affecting several of the hallmarks.

The causal approach we adopt—information transmission—helps us combine data and results from different types of studies on cancer. In this framework, our goal is to reconstruct the temporal process of cancer development by intercepting salient events in this process—this is the idea of the *transmission of information*. At the same time, this approach to causality gives us the flexibility to combine cues that are of different nature: the causal process of cancer development needs not to happen all at the same molecular level. Instead, we can meaningfully combine results coming from different types of studies. The methodology of the “meeting-in-the-middle” operationalizes at the design level this idea.

In spite of the limitations, we believe that what we propose may be a promising approach, both for the description

and evaluation of the existing literature on environmental causes of disease, and for novel exposome research.

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