

EDITORIAL

Neuroblastoma in childhood and its potential viral involvement: A webinar by the Paediatric Virology Study Group

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the unmoved mover (ὁ οὐ κινούμενον κινεῖ)

Aristotle

Abstract. Neuroblastoma represents the most common and lethal solid tumour of early childhood. In view of variations in genetic elements, MYCN amplification is certainly the most prominent genetic factor occurring in 1/4 or 1/5 of children with neuroblastoma; however, overall, the pathogenesis of neuroblastoma remains to be resolved. Rare, sporadic infections with Epstein-Barr, hepatitis C virus and varicella-zoster virus have been detected in children with neuroblastoma, while the presence of BK virus was initially claimed, but later falsified as a triggering factor for the development of high-risk neuroblastoma. The proposed model by Professor Ugo Rovigatti, Professor of Molecular Biology at the University of Florence in Italy, is based upon infection with micro-foci inducing virus and its potential tumorigenic role as trigger of i) high and persistent inflammation; ii) chromothripsis and genetic instability; and iii) *in vitro* cell transformation and *in vivo* carcinogenesis.

Modelling is still under-utilized and under-developed in clinical virology and cancer research; however, it is expected to play a significant role in the future, aiming to elucidate the cancer enigma. This article is based on a webinar on neuroblastoma in children, which was organised virtually on December 12, 2020, by the Institute of Paediatric Virology.

Introduction

What is the pathogenesis of neuroblastoma in childhood? How can the aggressiveness of high-risk neuroblastomas be interpreted? What is the contribution of viruses and viral infections in the pathogenesis of neuroblastoma in childhood? With what means is research trying to investigate the hypothesis of viral involvement in the pathogenesis of neuroblastoma in childhood? Will this hypothesis finally be proven false or true? What is the value of modelling in virology? What is the role of modelling in current and future cancer research? Professor Ugo Rovigatti, Professor of Molecular Biology in the Department of Experimental and Clinical Medicine at the University of Florence in Florence, Italy, tried to answer these questions during a webinar on neuroblastoma in children, which was organised virtually on December 12, 2020, by the Paediatric Virology Study Group (PMSG) of the Institute of Paediatric Virology based on the island of Euboea in Greece. Professor Ugo Rovigatti's CV is available at https://spandidos-publications.com/COVER_LEGENDS/ijo_58_3_cover_legend.pdf.

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Questions and answers

Question: First of all, Professor Ugo Rovigatti, thank you for your feedback and your wishes regarding the foundation of

the Institute of Paediatric Virology on the island of Euboea in Greece. What is neuroblastoma, what is its incidence in childhood and how is it staged?

Answer: Although neuroblastoma is a rare disease affecting ~10.5 children per million of total children aged 1-15 years, it is the most common solid tumour of early childhood, i.e., children of pre-schooling age. Therefore, it is only the sixth among paediatric tumours in terms of its frequency, but in view of the aggressiveness of its high-risk form, it causes a disproportionately high lethality, ~15% among paediatric cancers. The reason is the fact that high-risk neuroblastoma is in a greater part untreatable, although major progresses were recently made therapeutically. I will come back to this, but to give a general overview, it is as if there are two diseases. One is rather benign, where treatment gives good prognoses. There is also the early childhood form IV-S, before 1.5 years of age, which - although apparently aggressive and metastatic to several sites - spontaneously regresses, often without clinical intervention, i.e., without radio-/chemo-therapy. However, the more severe form in older children is very difficult to cure despite aggressive treatments and even autologous hematopoietic stem cell transplantation, treatments which, however, have been associated with survival improvements. Some recent success seems to be associated with so-called immunotherapy (IMT), in particular the usage of monoclonal antibodies, which target a glycosphingolipid: GD2. In this time-line and perspective of therapeutic improvements, the 2009 International Neuroblastoma Risk Group (INRG) Staging System (INRGSS) article was certainly a milestone (1), in the sense that i) it allowed the achievement of an internationally standardized diagnostic stratification of neuroblastoma cases; ii) most of all, the staging system was performed pre-operatively, differently from previous staging [International Neuroblastoma Staging System (INSS)], thus allowing much better interventions; and iii) an important impact in this classification is provided by the so-called image defined risk factors, which allow differentiation for example between stages L1 and L2. Imaging specialists and radiologists, therefore, hold key role in this new classification. Needless to say, INRGSS also needs and will be improved in the near future as different staging systems, such as the survival-tree regression (STR), and the least absolute shrinkage and selection operator (LASSO), are being compared by the Children's Oncology Group and other international organizations (2).

Question: How does the prognosis of high-risk neuroblastoma cases differ compared to other neuroblastoma cases or children with other neurogenic origin tumours?

Answer: I already alluded to the dramatic difference in terms of prognosis between high- and low-risk neuroblastomas. The question is very appropriate in the sense that it deals with the central-core of the neuroblastoma enigma and could also be summarized as: 'Why cells, which morphologically appear undistinguishable, as malignant small blue-round cells, behave so differently in terms of prognosis?' And this question intersects with our basic understanding of neuroblastoma, in the sense that the first molecular biology discoveries in the field certainly shed some light on this enigma. In particular, the seminal paper by Manfred Schwab working in Mike Bishop and Harold Varmus lab discovering the MYCN amplification (MYCNA) in high-risk neuroblastoma, but not in its less aggres-

sive forms, set the stage for this paediatric tumour present and future understanding (3). Furthermore, being this one of the first genetic aberrations discovered in cancer cells, it pioneered all future work in molecular oncology. We recently published an overview perspective on these issues: The shift from our cancer understanding in terms of 'clonal outgrowth', basis of radio-/chemo-therapy approaches to that of 'cancer gene network', basis of targeted gene therapies (TGTs), starts from discoveries such as the MYCNA in neuroblastoma (4). In 1984, Brodeur *et al* (5) initially demonstrated MYCNA as a marker of high-risk neuroblastoma and then several hundred clinical studies confirmed this as a hallmark of ominous prognosis (6). To conclude with neuroblastoma prognosis, this can be excellent in low-stage disease with a progression-free survival (PFS) and overall survival (OS) of $\geq 90\%$. However, in high-risk neuroblastomas, prognosis is only ~40% and in cases of recurrent or relapsing neuroblastomas, this figure goes down to 10%.

Question: The variation of clinical outcomes in neuroblastoma cases indicates distinct genetic and environmental factors affecting the development of this malignancy. Could you describe to us the main factors involved in its pathogenesis?

Answer: This impinges again on the enigmatic nature of neuroblastoma. Therefore, we can just be speculative and try to summarize a plethora of papers and investigations on this issue. Epidemiological work for decades could not identify among several potential carcinogens, pollutants and specific behaviours, one or some that could act as specific factors associated with its onset. Genetic and pedigree studies have also identified congenital mutations, which appear to be associated with rarer familial forms (7). However, these are the iceberg tip of neuroblastoma cases, which are mostly not-congenital and the aggressive ones are diagnosed after 18 months of age. Among genetic factors, MYCNA is certainly the most prominent, since it occurs in approximately one quarter of cases. I already described the important breakthrough of Schwab *et al* (3) of MYCNA in high-risk neuroblastoma. However, the problem of science - or its beauty depending on different opinions - is that as soon as you solve one important and interesting question, another or several ones pop out like mushrooms. MYCNA origin for example is difficult to be explained in biological and molecular terms. The genomic aberration appears to be sometimes 'huge'. For example, we have documented one high-risk neuroblastoma case, in which MYCN gene was amplified ~1,000 times. This means that the whole region of MYCN (called amplicon, typically large = one million base pairs - 1 MB - or even more) is amplified 1,000 times: In our genome of just >3 GB, this means that one third of this cell genome is totally aberrant (8). This is certainly difficult to explain in terms of random events, such as ephemeral point mutations, casually happening during DNA replications (9). It also strongly points towards natural selection for such a catastrophic event (10). For its origin, we have proposed a model based upon the micro-foci inducing virus (MFV) infection, since we can experimentally show that MFV infection of normal cells initially MYCN diploid modifies them into cells, which are transformed, tumorigenic and with higher MYCNA (100X). That epigenetic factors also play a relevant role in neuroblastoma was initially documented by induction of their differentiation with retinoic acid in the

study by Thiele *et al* (11). Several groups, including ours, have also documented the possible phenotypic transition/transformation of mesenchymal cells with stemness markers, into neuroectodermal clones sometimes with transformed cell features (12-14) and the group of Rogier Versteeg tentatively associated some of these phenotypes with a network of transcription factors (15). Additional alterations in telomerase or other epigenetics involved genes have also been described in high-risk neuroblastoma (16). However, in view of genetic elements variations, including mutations, deletions, amplifications, segmental chromosomal alterations etc., as well as alterations of epigenetic elements, the overall picture still looks as an unsolved puzzle (16). Using the science of logics that was born in Greece almost 2.5 thousand years ago, we are missing the understanding of the first mover. Aristotle, in his book 'Metaphysics', used to talk about 'the unmoved mover' (ὁ οὐ κινούμενον κινεῖ). The alternative explanation of alterations occurring randomly and cumulatively is extremely unlikely, also in view of the patients' very young age (9,10).

Questions: What are the genomic landscapes and how are they involved in the pathogenesis of high-risk neuroblastoma?

Answer: Genomic landscapes in neuroblastoma have been extensively studied, starting from the mentioned MYCNA present in 20-25% of cases. Some associations have been clearly identified. For example, MYCNA is typically associated with chromosome 1p deletions and 17q gains, while in another fraction of tumours the association is between 11q deletions, the alpha thalassaemia mental retardation X-linked mutations and associated death-associated protein 6 loss: The so-called telomere maintenance mechanism or alternative lengthening of telomeres (16). However, the definition of such alterations has been often elusive. For example, at the beginning of the 1990s, a real hunt had started for the identification of the gene or genes, which are present in the chromosome 1p region, which is often lost in high-risk neuroblastomas, generally with MYCNA. Therefore, this could be most likely explained by the loss of a tumour suppressor gene (TSG). Despite several publications, efforts and competitions between the major groups working on both sides of the Atlantic, as well as in Japan, the 1p TSG has not materialized so far, although some interesting candidates were proposed (6). The neuroblastoma field is more realistically now directing its attention towards understanding how such genetic/genomic landscapes are generated and most of all why. In fact, although some of the described alterations are also targetable by TGT, mutations in high-risk neuroblastomas appear to be so numerous and with relatively low frequency to impede utilization by so called precision medicine (16,17). This becomes today a general theme of cancer genetics and genomics, that we have recently addressed in two publications (4,10). In other words, the idea of targeting mutations specifically present in a particular type of cancer - although good in theory - is invalidated by tumour heterogeneity (TH), a concept which is now becoming more recognized in cancer biology and molecular biology (4,16).

The other essential concept to explain TH is the Darwinian selection. Cancer cells are extremely prone to change their genetic make-up, in order to respond to environmental selection, such as chemotherapy or TGT (10). In high-risk neuroblastomas, not only inter-TH, but especially intra-TH plays an essential role, as mutations in the RAS-MAPK

pathway often appear in relapses (18). In these cases, therefore, mutations more than an essential role by themselves appear to have a consequential role. They seem to be a consequence of the Darwinian selection (10). This may also be a more general problem of cancer genomic landscapes and obviously the important question then arises: Which are the Darwinian selection forces or factors behind (4)? In this respect, it may be instructive to read again the article by Gatenby *et al* (19).

Question: How frequent are cancer clusters of neuroblastoma cases in specific geographic areas over a period of time and how could they be related to the pathogenesis of neuroblastoma?

Answer: Clusters of neuroblastoma cases are rather rare for at least two reasons. Firstly, neuroblastoma is a rare childhood malignancy, with a frequency ~50 times lower than paediatric leukaemia. Secondly, although leukaemia clusters have been described in several instances, less is known about neuroblastoma clusters. Recently, neuroblastoma space time clusters have been reported in the USA, Argentina and Spain (16). Another report from the UK described several instances of neuroblastoma time-clusters (20). For these researchers, such temporal clusters, detected by studying 227 cases during a period of 44 years, appear to be 'mini-epidemics' that happen often and in several different locations (20). The reason of the appearance of cancer-clusters, especially in children, has been often and heatedly debated. They seem to emerge in children rather than in adults, in view of the immune system, which is still underdeveloped earlier in life (21). However, such clusters are especially evident and have been more extensively studied in paediatric leukaemia, which is more frequent. In such situations, childhood leukaemia incidence is, for a limited time, several folds higher than national and international average (21). Most debated hypotheses for leukaemia clusters are the 'population-mixing' and the 'delayed infection', respectively by Kinlen (22) and Greaves (23). The first one hypothesizes that an infectious agent is carried by an exposed population - typically from densely inhabited areas. Isolated and unexposed groups will lack herd immunity against such an agent. Therefore, the immigration of exposed populations towards more isolated regions will generate such mini-epidemics or leukaemia clusters (22). The second hypothesis is more general and vague. It suggests a higher risk for segregated childhood populations, for example, groups with higher income, but it does not clarify whether one specific or several possible infections may cause the trigger; the second hypothesis is privileged by Greaves (23). However, both hypotheses agree on the presence of and causality by an infectious agent, either one X-virus according to Kinlen (22) or any possible virus - but also other agents such as bacteria - according to Greaves (23). Our findings on the MFV associated to a neuroblastoma cluster is in agreement with the hypothesis of Kinlen (22), although it could also be an example of delayed infection. However, the MFV model more clearly explains how and why genetic/genomic aberrations are generated in cancer cells.

Question: The viral aetiology of neuroblastomas has been proposed very early, almost 50 years ago, but it has not been proven. Which oncogenic or non-oncogenic viruses have been

detected in cases with neuroblastoma up to now? How do you explain the presence of these viruses in neuroblastoma cases? *Answer:* The fact that neuroblastic tumours may be associated with previous viral infection has been discussed throughout the years, starting from Robert Bolande's definition of neurochristopathies and their associated tumours 50 years ago (24). However, it should be clarified that there are at least three different types of studies and approaches to this problem. Firstly, certain laboratories, for example, have just detected infections, which are present in neuroblastoma patients. This has been even utilized to neuroblastoma patients' advantage. The new chimeric antigen receptor T-cell technology, which directs T-cells against an appropriate tumour target, took advantage of neuroblastoma cases with concomitant Epstein-Barr virus (EBV) infections. By co-targeting T-cells against EBV capsid antigens and GD2 glycosphingolipid, a much stronger response could be elicited (25). A few instances of concomitant infections have been described: EBV, hepatitis C virus (HCV), varicella-zoster virus (VZV), etc.

Secondly, another approach has been taken by researchers, which hypothesized that a certain family of viruses could be associated with high-risk neuroblastoma - Herpes family viruses in one project, Polyoma type viruses, such as BK virus (BKV) in another, etc. These approaches typically detect some positivity in a limited percentage of cases. This could be exemplified by the detection of BKV presence in neuroblastoma cells obtained by Flaegstad *et al* >20 years ago (26). Obviously, such studies must then convince us, the scientific community, that the presence of that particular virus is significantly higher or more frequent in neuroblastoma patients. In the case of BKV, differences were rather slim and the project was eventually dropped (27).

Thirdly, our approach for detecting, isolating and studying MFV has been completely different. We started by studying in the laboratory tumours from a cancer-cluster of neuroblastoma cases in Morgan City (LA, USA) (13). In view of a strong TH, we then tested the hypothesis that an infectious agent was present by adding ultra-filtered supernatants to normal cells. This finally led to electron microscopy studies, which disclosed the presence of a cytoplasmic virus (13). We have then employed several experimental systems both *in vitro* and *in vivo*, which document the transforming capability and tumorigenicity of MFV. Furthermore, the molecular mechanism was investigated leading to the qualified conclusion that MFV can induce molecular aberrations similar to those present in the original tumours, i.e., MYCNA (8,13). This is particularly compelling, since origin of such genomic aberrations is an unsolved enigma not only of the molecular biology of neuroblastoma, but also of cancer cells in general (8,10).

Question: Recent studies have proposed Zika viral therapy as an adjunctive treatment for neuroblastoma by targeting tumour cells that can lead to recurrent disease and treatment failure. We would like your comment on this possibility.

Answer: Zika virus has become a health concern in the past few years, especially in view of their infections during pregnancy, which can lead to foetal/neonatal microcephaly. This was particularly alarming in 2016, when a Zika virus epidemic was spreading throughout Brazil and South America during the past Olympic Games. The idea to employ now Zika virus against neuroblastoma or other neuroectodermal

tumours, such as glioblastomas, is based on the Zika virus targeting of neural cells (28). However, the general idea of employing viruses for targeting tumours and tumour cells is a very old one. We were previously discussing about a viral hypothesis for neuroblastoma aetiology, but the proposal of employing so-called oncolytic viruses for cancer therapy dates back at least 70 or even 80 years. There are many examples of viro-therapy attempts in these 70-80 years: from hepatitis viruses to EBV, from West-Nile virus (WNV) to adenovirus and more recently: paramixo-, herpes, picorna- and pox-viruses as well as enteroviruses. Unfortunately, no real candidate has finally arisen from these studies, in view of great toxicities and other problems. For all these reasons, I am rather sceptical about Zika virus attempts in neuroblastoma or other neuroectodermal malignancies. We should not forget that malignancies in general, and especially neuroblastoma, are based upon transformation of stem cell targets. Zika virus lytically also infects neural stem cells, thus causing microcephaly of foetuses and neonates. Therefore, one can always imagine a scenario, in which defective Zika virus particles could even be associated with transforming/malignant effects. I honestly believe that excessive manipulations of dangerous pathogens are not particularly useful unless very specific and well demonstrated instances indicate so. However, there is not so far - despite experimental studies for >70 years - an acceptable candidate for oncolytic therapy, especially for high-risk neuroblastomas. A similar philosophy may be deduced and should be applied on the extensive research on pathogenic, lethal and pandemic coronaviruses (or influenza viruses) performed between 2009 and 2019, since it has not produced so far important treatments, nor was capable of preventing - as we are all personally and dramatically experiencing today - one of the worst pandemic outbreaks in decades. We also tend to forget that a sizeable portion of us, as scientists, with Simon Wein-Hobson as one of the leading most outspoken figures, strongly opposed such gain-of-function experiments [Rey *et al* (29)]. In conclusion, any intervention with biological agents should be carefully monitored and evaluated with the strictest scientific criteria. If there is no evidence of scientific or clinical advantage, it should be abandoned.

Question: Could you further describe to us your MFV model and its involvement in aggressive neuroblastoma genetic/genomic aberrations?

Answer: I have already partially described the MFV model. So, I will add some further essential elements, which may be helpful for your PVSG. Firstly, I will analyse the clear presence of clusters of neuroblastoma cases; secondly, the peculiar aspects of the experimental animal models and thirdly, the presence of very high MYCNAs in initial tumour samples and their study in derived models.

So, let's start with the first. Although rarer, neuroblastoma clusters have been described in the past in different continents and regions. In the UK, there is evidence in favour of temporal clusters, but most of the other instances are based on space-time clusters (Argentina, Spain; Florida, Louisiana, USA). I have already briefly described the theories on cancer-cluster onset, mostly based on childhood leukaemia studies. In our model as well as in other instances - for example in the extensively studied

clusters of Sellafield (UK) and of Fallon (NE, USA) - presence of a specific virus [X-virus according to Kinlen (22)] better explains the epidemiological data. Was the Morgan City cluster isolated? Probably not, as paediatricians alerted us that excess cases were diagnosed throughout Southern Louisiana and in the neighbour State of Texas in 1987-1988, but we were able to study only this isolated cluster in Morgan City (LA, USA) (13).

Secondly, since the beginning of this isolation and discoveries, it has been of paramount importance for us to develop animal as well as cell culture models. The first animal models were based on rats, while later we evaluated carcinogenesis in nu/nu mice (13,30). The great interest of rat models was based on their capability to recapitulate several facets of paediatric neuroblastomas. Not only neuroblastoma tumours appeared in all the litters from experimentally infected mothers, but other aspects, such as the opsoclonus myoclonus syndrome (OMS), ataxia, the raccoon eyes typical of children with neuroblastoma at diagnosis, watery diarrhoea due to the vasoactive intestinal peptide, etc. (13,30).

And thirdly, the dramatic presence of MYCNA at a level (1,000X amplification) only rarely described was another red flag for these tumours. Amplification, however, seemed to disappear when growing tumour cells in tissue culture and rapidly so (after 3-4 passages) (13). In trying to rationalize what was happening *in vitro*, it was realized that two components were present *in vitro*. Proliferating cells had a flat, mesenchymal and Schwann-like appearance (S cells) and grew attached to the bottom of the flasks, while on top of them micro-foci of small-round-blue cells with neural markers (N cells) could form. Only N cells of micro-foci displayed high/very high MYCNA, while S cells remained MYCN diploid (13). This induction of oncogene amplification has been studied in subsequent years as a model for the genesis of cancer-specific aberrations, possibly not just for neuroblastoma (8,30).

Question: How could this model be used in the understanding of neuroblastoma pathogenesis? How could this model be involved in the novel therapies against aggressive neuroblastoma?

Answer: I will consider these two questions separately and consequentially. Neuroblastoma is a puzzle made with plenty of tale stones for which we still lack a solution. Neuroblastoma is also associated with a number of uncommon if not paradoxical facets, such as the OMS or racoon eyes syndrome mentioned before, the MYCNA which is rather frequent being present in 20-25% of cases and the peculiar behaviour of the intriguing subset of neuroblastoma named IV-S disease. Our MFV model has the potential to accommodate in a logical framework most if not all of these aspects. Take the IV-S disease as an example. Since the seminal study by D'Angio *et al* (31), we know that neuroblastoma in neonates or very young children can also appear as a widespread and metastatic disease - throughout the body, in blood, in bone marrow but not inside bones - and yet suddenly and spontaneously regresses before 18 months of age (the threshold was considered before at 1 year). This phenomenon is so dramatic and reproducible that clinicians typically wait to treat IV-S patients with radio-/chemo-therapy, since they know that nature will spontaneously find the therapy. Our model with MFV offers a simpler explanation. If this is caused by an infecting virus that *Homo sapiens* is used to live

with, it is quite possible that the slowly developing immune system eventually gets rid of the problem. I am particularly thinking about the cellular immunity (natural killer cells, specialized T-cells, etc.), which ontogenically appears at around 1 year of age. But it is particularly in the area of molecular genetics that our model has great potential, since we have demonstrated in several experiments and publications that MFV infections cause MYCNA, also at dramatic levels (100X) with certain cell lines, such as SK-N-AS, VA-N-BR, etc. (8,13,30). Genetic aberrations are still another enigma of high-risk neuroblastoma and cancer cells in general for which the MFV model provides a potential explanation, as we have discussed in papers about cancer modelling (4,10,16).

Cancer modelling takes into account all aspects of a particular theory and even its consequences, for example its therapeutic implications. This can even become a method for invaliding or falsifying a particular hypothesis, as we have recently critically discussed TGTs and their therapeutic implications (4). In high-risk neuroblastomas, there has been a slow progress, since a sizeable portion of cases still has recurrences and dies - up to 60%. Unfortunately, this figure is even higher in relapsing/progressing cases since survival becomes only 10%. A few decades ago, one of the first form of cancer IMT was initiated for high-risk neuroblastomas at the Memorial Sloan Kettering Cancer Center (MSKCC) by Nai-Kong Cheung and Brian Kushner with a monoclonal antibody against the glycosphingolipid GD2. Several clinical trials showed that passive anti-GD2 IMT provides a 20% PFS improvement in these patients (32). However, the reasons for this excellent result are not very clear, particularly since extensive knowledge has been accumulated on genomic landscapes and genetic markers for high-risk neuroblastomas, but no specific marker has been associated with GD2 presence/persistence and with its therapeutic targeting successes (16). In a recent publication in *Cancer Letters*, I proposed an alternative explanation (16). The MFV model shows that genomic alterations, such as MYCNA, are induced by MFV infections, so that this could be the cause or one of the triggers of the disease. A clear link between GD2 and MFV could be envisaged by carefully analysing the sialic acid receptors for this family of viruses, i.e., the *Reoviridae*. Since another similar glycosphingolipid, GM2, is the receptor for the type 1 Lang strain, it is hypothesized that GD2 is the receptor for MFV and similar *Reoviridae* (16). This proposal and interpretation are strengthened by recent clinical data from the study by Kushner *et al* (33) at MSKCC. The IMT treatment of MYCNA-positive cases provides excellent survival results with PFS of 82% and OS of 94% (33). These results are rather similar to what can be achieved in low stage disease, not in cases which are usually considered as high-risk neuroblastomas (33).

Question: How is your model evaluated in the following years?

Answer: There are several ways to evaluate the possible relevance and especially heuristic consequences and therapeutic applications of the MFV model. Before indicating at least some of the steps that I would consider essential for evaluating the MFV model, I will also briefly comment on how to practically perform such an evaluation. Since I will retire from active teaching duties from the University of Florence at the end of this academic year, I am planning to invest then all of my working time into research, especially on the MFV model.

I am also presently considering moving to different countries, in view of the fact that research in Italy is very poorly funded and appears to be dominated by a patronizing system - at least at the university level where I worked for three decades. This means that research projects are often funded more on the basis of personal liaisons and friendships rather than for real scientific merits. I am presently considering five different locations: Two in the USA and three in Europe, where I could possibly relocate. There are at least four major themes, which will be investigated in order to evaluate the MFV model.

Firstly, one will be the extension of my most recent article and presentations on anti-GD2 passive IMT (16). As described in my previous answer, the striking effects of anti-GD2 IMT do not find an obvious explanation with current studies on genomic landscapes, but could be explained by the qualified hypothesis of GD2 glycosphingolipid being associated with MFV receptor recognition and cell entrance. Dinutuximab and other monoclonals utilized for therapy will be employed initially in *in vitro* systems to assess effects on MFV infection. Subsequently, the previously described animal systems will be utilized and when indicated we will also investigate patient specimens from current anti-GD2 trials or treatments.

Secondly, the core-part of this project will be the molecular and cellular evaluation of this model. As previously described, we know that MFV infections can elicit experimental MYCNA up to 100-fold in SK-N-AS, VA-N-BR, etc. Besides testing additional cell lines, also from different paediatric and adult tumours, we want to investigate and better understand the mechanism of such catastrophic events. One explanation, which is being evaluated today is whether extensive genomic aberrations may be associated with chromothripsis or similar events of chromosome shattering and repasting (16). This was discovered in approximately one out of five neuroblastoma cases in the study by Molenaar *et al* (34), and we have evidence that MFV infection could cause it (10). Stem cells will be also investigated as targets of MFV induced aberrations and malignant transformation (16).

Thirdly, I previously alluded to the very exciting results of Kushner *et al* (33) at MSKCC and similar results were obtained at St. Jude and by the International Society of Paediatric Oncology European Neuroblastoma Group (SIOPEN), a cooperative group that is committed to paediatric neuroblastoma research (16). These data suggest that the excellent survivals are indeed linked to peculiar cases with MYCNA, which raises the possibility of MFV infections, associated with anti-GD2 IMT. Therefore, IMT could be particularly efficacious for a target involving MFV. We will investigate how many of such targets could be envisaged and tested for efficient therapies in either high-risk neuroblastoma or additional paediatric and adult tumours.

And lastly, in testing this MFV model, we will expand our analysis to additional paediatric tumours, such as medulloblastomas, lymphomas - especially of Burkitt's lymphoma (BL) type-, and leukaemias, as well as adult ones, where MFV-like viruses have been either isolated or hypothesized, such as small-cell lung cancer - similar to neuroblastoma-, prostate cancer, lymphomas, breast cancer, etc.

Question: What is the value of modelling in virology? Could you give us examples?

Answer: Modelling in virology has an exceptional value. In fact, virology could not exist as it is today without virology model-

ling. There are myriads of examples, but let's concentrate only on outstanding virological discoveries, which were awarded a Nobel Prize in Medicine. Max Theiler was awarded the prize in 1951 for yellow-fever first vaccine; John Enders, Frederick Robbins and Thomas Weller for isolating and growing polio-virus in tissue cultures - this led to polio vaccines; Peyton Rous was awarded the prize in 1966 for his discovery of a cancer-causing virus, now known as the Rous sarcoma virus; Renato Dulbecco, David Baltimore and Howard Temin were awarded the prize in 1975 for their work on oncogenic viruses (Polyoma/SV40 and retroviruses); Baruch Blumberg in 1976 for his work on hepatitis B; Mike Bishop and Harold Varmus in 1989 for their discovery of viral and cellular retroviral oncogenes. In 2008, Harald zur Hausen was awarded the prize for his discovery of human papillomaviruses (HPVs) cervical carcinogenesis (35) together with Françoise Barré-Sinoussi and Luc Montagnier for discovering human immunodeficiency virus (HIV). Similarly, very appropriate was last year, 2020, the prize awarded to Michael Houghton, Harvey Alter and Charles Rice for their identification, characterization and growth of HCV, allowing screening and vaccination. In all these instances, virological modelling was extensively utilized to reproduce under experimental conditions both *in vitro* and *in vivo* essential facets of the infections, thus leading to major life-savers, such as specific vaccines. If you think about it, the discoveries of Harald zur Hausen, Baruch Blumberg, and the polio and HCV trios - just to mention some groups - have saved or cured the lives of hundreds of thousands of people. Our virological discovery of a novel virus in paediatric cancers and especially in cancer-clusters of childhood is more modest in terms of frequency, but scientifically certainly not unimportant and I want to defend it. I think there are three aspects of our viral model, which still deserve attention, because they were ignored or not sufficiently emphasized.

We were probably not the first to identify in animal, *in vitro* or *in vivo* models the presence of viruses like MFV. From the end of the 1960's, Elisabeth Gateff, then at the University of Freiburg and also collaborating with the Deutsches Krebs Forschung Zentrum in Heidelberg in Germany, described with other colleagues, similar viral particles in tumours and stem cells of *Drosophila melanogaster* (DM). Her findings are quite interesting, because the DM particles, whose size is quite different from the ones isolated by us in neuroblastoma or paediatric lymphoma, were finally identified as Reovirus particles in 1980 (36). Furthermore, in all the instances in which DM particles were isolated, the tissue of origin was either malignant - for example a DM brain tumour - or were cultures of stem cells. Therefore, this is another characteristic feature of MFV and similar viruses. Differently from HPVs, which actively replicate only in differentiated epithelium, MFV-related viruses (MFRVs) need to infect stem cells or cancer stem cells.

There was another instance in which these types of viruses were identified and then the notion was somehow 'dropped', during the chase for the 'equatorial belt virus'. The presence of a virus was hypothesized by the great physician, Denis Burkitt, who noticed the appearance of paediatric lymphomas, typically in the head-neck region, which seemed to follow a geographic and altitude pattern around the equatorial Africa. The ensued race for discovering if/which virus was responsible led not only

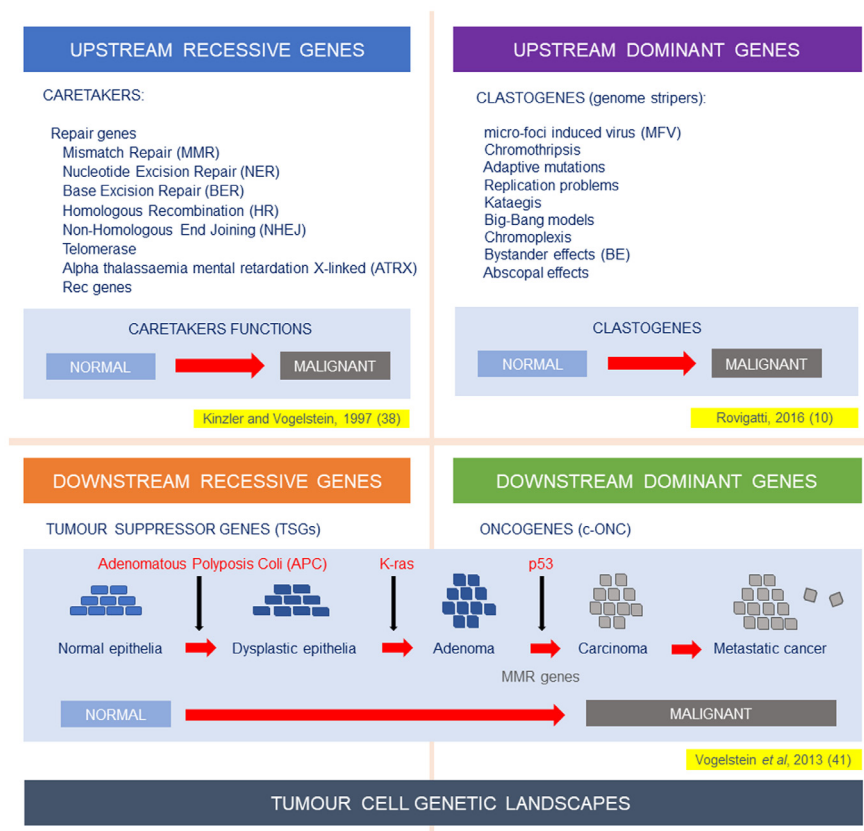


Figure 1. Distinction between downstream and upstream genetic elements in our cancer genetic landscape. Only upstream recessive elements (upper left panel) have been previously recognized. Several evidences suggest that also upstream dominant elements (upper right panel) are indeed present and active during cancer onset, progression and relapses (4,10,16). The illustrated figure parts have been adapted from previous studies (10,38,41) as indicated.

to the discovery of EBV by Anthony Epstein and his assistant Ivonne Barr, who received BL samples in London. At the same time, several isolations of novel Reoviridae family viruses were obtained by Thomas Bell and colleagues at the Imperial Cancer Research Fund (ICRF) in Uganda. These discoveries were documented in prestigious journals, such as the *British Journal of Medicine* (37), but finally dropped in favour of EBV by the scientific community. Our re-discovery of MFRVs in cases of BL in Switzerland - where it is difficult to object that Reoviruses would be isolated in view of poor hygienic conditions - strongly argues in favour of an important role for this family of viruses in BL pathogenesis (30). Furthermore, as it was indicated in the original papers in the 1960s, we also discovered a few cases with presence of both EBV and MFV. As previously indicated for the replicative difference between HPVs and MFV, the issue of co-infection should be investigated again, since different families of viruses could have complementary roles in malignant transformation. EBV for example appears to be an immortalizing virus while MFV behaves as a clastogenic and transforming virus.

The general and take-home message of this virological modelling for this family of viruses is that MFV/MFRVs are 'normal-passengers' or viruses persistently infecting *Homo sapiens* - also according to the epidemiological data presented in the study by Kinlen (22), probably harbouring inside stem cells (36). It should be clarified why certain isolates are associated with grave malignancies such as neuroblastomas and lymphomas. However, something similar is clearly happening

with high-risk HPVs, such as HPV 16, 18 and 31 clearly linked to cervical cancers (35).

Question: What is the value of cancer modelling in the understanding of cancer pathogenesis and therapeutic interventions?

Answer: Cancer modelling refers to a general re-analysis, or sometimes meta-analysis, of all the data we have in favour or against a particular hypothesis to explain cancers and especially human cancers. We have used this term for the first time in a longer review article finally published in 2015 (4), also in view of my criticism of the modelling present at that time. I have argued in that paper, and I am still convinced today, that the falsification of a model in cancer research does not come or originate only from experiments performed in the laboratories, but also by the clinical practice that applies such model for treating cancer patients. In other words, if the logical consequences of a model lead to a specific type of therapy and this therapy is not cancer-curative, this should logically lead to the falsification of the model itself. We should therefore distinguish three phases in this reassessment of cancer modelling, which however follow the current trends of cancer research.

My criticism in 2015 was qualified by an extensive analysis of the pitfalls of TGTs, at that time the most accepted cancer therapies. I argued that, as chemotherapy before was based upon interpretation of cancer as 'clonal outgrowth', TGT stands upon a vision of malignancies as derangements of 'gene networks'. We know today that clonal outgrowth is incorrect, since it has been shown that cancer is dominated by

TH, of which intra-TH drives continuous expansions of new clones, essentially created by new mutations accrual and by selection. This obviously leads to selection of chemo-therapy resistant clones. However, also the 'gene network' hypothesis is invalidated: i) Not only by intra-TH which obviously affects genes and their expression as first targets; but also ii) by what appears to be a general misconception of the model that has emphasized only final or landscape events of cancer genomes. This concept becomes rather obvious, if one analyses instead functions, which are considered upstream from such genomic landscapes. In Fig. 1 (upper left panel), I represent what was described till 2016, i.e., the recessive function of caretakers described by Kinzler and Vogelstein (38) several years before. However, it was becoming clear that novel functions should be hypothesized as present upstream of the genomic landscape. They ought to be dominant and clastogenic (Fig. 1, upper right panel).

Alternative modelling needs to be proposed, to overcome the difficulties of hallmarks of cancer (HoC) and TGT models. What appears to be their common denominator is the incapability of solving the TH conundrum. In 2015, we proposed to utilize additional models instead of the only one which has dominated for 20 years (HoC) (4). This strategy follows also the example of our colleagues, physicists and astrophysicists, who have proposed several different models. It was therefore proposed that by following different alternative or complementary modelling, we may reach more rapidly the solution of the cancer enigma (4). In 2016, following the described diatribe over origin and mechanisms of TH, the original scheme was modified by adding dominant functions, which actively cause clastogenic activities on the genomic landscapes downstream: Both oncogenes and TSGs (Fig. 1, bottom panels). Clear examples were already present with chromothripsis, kataegis and chromoplexis and examples of Big-Bang models from human colorectal cancers (10,39). Therefore, the MFV model appears to be probably the first described example of such upstream-dominant activities (10,13,16). Its natural history of infections - occurring very early in ontogenesis, even during the first months of life, its persistence with life-long immunity and associated clastogenic and mutagenic events, often through chromothripsis, render the MFV model a particularly interesting one, which should be further elaborated and studied in the future (10,16).

Question: So, where do we go from here? What should be the future steps?

Answer: In 2018, cancer modelling history was somehow changed when the Nobel Prize for Medicine was assigned to James Allison and Tasuku Honjo for their fundamental discoveries on inhibitors of immune checkpoints or negative regulators. Heralded by lay press and editorial commentaries, these discoveries announced a progressive shift today toward IMT approaches. It is, however, difficult to believe that IMT will be - as it is now - the panacea for all human cancers. In fact, many of the expressions of excessive enthusiasm for IMT today are reminiscent of the hype surrounding TGTs and targetable or 'actionable' genes (40) almost 20 years ago. Since I am wearing white hair now, I could suggest some caveats and essential experiments in order to clarify today's picture. Although the therapeutic benefits of anti-PD1, PDL1, CTLA4, etc., are

in some cases undeniable and obviously very welcomed, we should clarify what such activities mean in cellular-molecular terms. In other words, we should clarify their targets and their meanings. My recent article in *Cancer Letters* on the IMT passive targeting of GD2 in paediatric high-risk neuroblastoma could be an example (16). Since next-generation sequencing studies and genetic/genomic data do not show any clear correlation with GD2 and since Kushner *et al* (33) demonstrated excellent results by IMT in children with MYCNA, the qualified hypothesis was made that GD2 acts as recognition-receptor for MFV (16). Similarly, in most of the therapeutic successes of today's 'inhibitors of negative immune regulators', we just know that there is an increased action of the immune system, but we ignore the target(s).

We should be also aware of our previous experiences. TGTs probably relied on an 'incomplete model' (4), where the analysis was frozen at the lower level of genetic mutations/aberrations (Fig. 1); these are called tumour cell genetic landscapes. These landscapes are extremely mobile and variable and they also depend on upstream elements, which are actively and dominantly hitting the genome. These 'Aristotle's first mover(s)' should be better identified, studied and therapeutically approached, as it also depends on them the whole well-being of our genome.

Finally, we should not forget that in all previous instances, we have caused and we are still causing our oncologic patients to become cancer-therapies addicts. It is clear now that most of the mutations evidenced in radio-/chemotherapy treated patients are due to the specific treatments (radio-/chemotherapy) following Darwinian mechanisms. Similar effects are elicited and evident in TGTs. Think twice before starting creating again an army of cancer patients addicted to immunotherapeutic drugs! This is also why it is so essential to unveil TH and cancer 'first movers'. The antibiotics example is paradigmatic and should lead us in this search.

Question: Thank you very much for participating in our webinar.

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References

- Monclair T, Brodeur GM, Ambros PF, Brisse HJ, Cecchetto G, Holmes K, Kaneko M, London WB, Matthay KK, Nuchtern JG, *et al*; INRG Task Force: The International Neuroblastoma Risk Group (INRG) staging system: An INRG Task Force report. *J Clin Oncol* 27: 298-303, 2009.
- Naranjo A, Irwin MS, Hogarty MD, Cohn SL, Park JR and London WB: Statistical Framework in Support of a Revised Children's Oncology Group Neuroblastoma Risk Classification System. *JCO Clin Cancer Inform* 2: 1-15, 2018.
- Schwab M, Alitalo K, Klempner KH, Varmus HE, Bishop JM, Gilbert F, Brodeur G, Goldstein M and Trent J: Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. *Nature* 305: 245-248, 1983.
- Rovigatti U: Cancer modelling in the NGS era - Part I: Emerging technology and initial modelling. *Crit Rev Oncol Hematol* 96: 274-307, 2015.
- Brodeur GM, Seeger RC, Schwab M, Varmus HE and Bishop JM: Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. *Science* 224: 1121-1124, 1984.
- Matthay KK, Maris JM, Schleiermacher G, Nakagawara A, Mackall CL, Diller L and Weiss WA: Neuroblastoma. *Nat Rev Dis Primers* 2: 16078, 2016.
- Mossé YP, Laudenslager M, Longo L, Cole KA, Wood A, Attiyeh EF, LaQuaglia MJ, Sennett R, Lynch JE, Perri P, *et al*: Identification of ALK as a major familial neuroblastoma predisposition gene. *Nature* 455: 930-935, 2008.
- Rovigatti U: Chronic Fatigue Syndrome (CFS) and Cancer Related Fatigue (CRF): Two 'fatigue' syndromes with overlapping symptoms and possibly related aetiologies. *Neuromuscul Disord* 22 (Suppl 3): S235-S241, 2012.
- Tomasetti C and Vogelstein B: Cancer etiology. Variation in cancer risk among tissues can be explained by the number of stem cell divisions. *Science* 347: 78-81, 2015.
- Rovigatti U: The Long Journey of cancer Modeling: Ubi Sumus? Quo Vadimus? *Mathew J Cancer Sci* 1: 1-5, 2016.
- Thiele CJ, Reynolds CP and Israel MA: Decreased expression of N-myc precedes retinoic acid-induced morphological differentiation of human neuroblastoma. *Nature* 313: 404-406, 1985.
- Ross RA, Biedler JL and Spengler BA: A role for distinct cell types in determining malignancy in human neuroblastoma cell lines and tumors. *Cancer Lett* 197: 35-39, 2003.
- Rovigatti U: Isolation and initial characterization of a new virus: Micro-Foci inducing virus or MFV. *C R Acad Sci III* 315: 195-202, 1992.
- Furlan A, Dyachuk V, Kastriti ME, Calvo-Enrique L, Abdo H, Hadjab S, Chontorotzea T, Akkuratova N, Usoskin D, Kamenev D, *et al*: Multipotent peripheral glial cells generate neuroendocrine cells of the adrenal medulla. *Science* 357: eaal3753, 2017.
- van Groningen T, Koster J, Valentijn LJ, Zwijnenburg DA, Akogul N, Hasselt NE, Broekmans M, Haneveld F, Nowakowska NE, Bras J, *et al*: Neuroblastoma is composed of two super-enhancer-associated differentiation states. *Nat Genet* 49: 1261-1266, 2017.
- Rovigatti U: The glycosphingolipid GD2 as an effective but enigmatic target of passive immunotherapy in children with aggressive neuroblastoma (HR-NBL). *Cancer Lett* 503: 220-230, 2021.
- Fletcher JI, Ziegler DS, Trahair TN, Marshall GM, Haber M and Norris MD: Too many targets, not enough patients: Rethinking neuroblastoma clinical trials. *Nat Rev Cancer* 18: 389-400, 2018.
- Elefeld TF, Oldridge DA, Bernard V, Koster J, Colmet Daage L, Diskin SJ, Schild L, Bentahar NB, Bellini A, Chicard M, *et al*: Relapsed neuroblastomas show frequent RAS-MAPK pathway mutations. *Nat Genet* 47: 864-871, 2015.
- Gatenby RA, Gillies RJ and Brown JS: Of cancer and cave fish. *Nat Rev Cancer* 11: 237-238, 2011.
- Muirhead CR, Tweddle DA, Basta NO and McNally RJ: Temporal clustering of neuroblastic tumours in children and young adults from Northern England. *Environ Health* 14: 72, 2015.
- Heath CW Jr: Community clusters of childhood leukemia and lymphoma: Evidence of infection? *Am J Epidemiol* 162: 817-822, 2005.
- Kinlen L: Childhood leukaemia, nuclear sites, and population mixing. *Br J Cancer* 104: 12-18, 2011.
- Greaves M: Infection, immune responses and the aetiology of childhood leukaemia. *Nat Rev Cancer* 6: 193-203, 2006.
- Vega-Lopez GA, Cerrizuela S, Tribulo C and Aybar MJ: Neurocristopathies: New insights 150 years after the neural crest discovery. *Dev Biol* 444 (Suppl 1): S110-S143, 2018.
- Pule MA, Savoldo B, Myers GD, Rossig C, Russell HV, Dotti G, Huls MH, Liu E, Gee AP, Mei Z, *et al*: Virus-specific T cells engineered to coexpress tumor-specific receptors: Persistence and antitumor activity in individuals with neuroblastoma. *Nat Med* 14: 1264-1270, 2008.
- Flaegstad T, Andresen PA, Johnsen JI, Asomani SK, Jørgensen GE, Vignarajan S, Kjuul A, Kogner P and Traavik T: A possible contributory role of BK virus infection in neuroblastoma development. *Cancer Res* 59: 1160-1163, 1999.
- Giraud G, Ramqvist T, Pastrana DV, Pavot V, Lindau C, Kogner P, Orrego A, Buck CB, Allander T, Holm S, *et al*: DNA from KI, WU and Merkel cell polyomaviruses is not detected in childhood central nervous system tumours or neuroblastomas. *PLoS One* 4: e8239, 2009.
- Mazar J, Li Y, Rosado A, Phelan P, Kedarinath K, Parks GD, Alexander KA and Westmoreland TJ: Zika virus as an oncolytic treatment of human neuroblastoma cells requires CD24. *PLoS One* 13: e0200358, 2018.
- Rey F, Schwartz O and Wain-Hobson S: Gain-of-function research: Unknown risks. *Science* 342: 311-311, 2013.
- Rovigatti U, Tam A, Piccin A, Colonnato R and Sordat B: Preliminary Characterization of a New Type of Viruses Isolated from Paediatric Neuroblastoma and Non-Hodgkin's Lymphoma: potential Implications for Aetiology. In: *Proceedings of the International Conference on Childhood Leukaemia*, London, section P1-18, ppI-IV, 2004.
- D'Angio GJ, Evans AE and Koop CE: Special pattern of widespread neuroblastoma with a favourable prognosis. *Lancet* 1: 1046-1049, 1971.
- Yu AL, Gilman AL, Ozkaynak MF, London WB, Kreissman SG, Chen HX, Smith M, Anderson B, Villablanca JG, Matthay KK, *et al*; Children's Oncology Group: Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. *N Engl J Med* 363: 1324-1334, 2010.
- Kushner BH, LaQuaglia MP, Modak S, Wolden SL, Basu EM, Roberts SS, Kramer K, Yataghene K, Cheung IY and Cheung NV: MYCN-amplified stage 2/3 neuroblastoma: Excellent survival in the era of anti-GD2 immunotherapy. *Oncotarget* 8: 95293-95302, 2017.
- Molenaar JJ, Koster J, Zwijnenburg DA, van Sluis P, Valentijn LJ, van der Ploeg I, Hamdi M, van Nes J, Westerman BA, van Arkel J, *et al*: Sequencing of neuroblastoma identifies chromothripsis and defects in neuritegenesis genes. *Nature* 483: 589-593, 2012.

35. zur Hausen H: Papillomaviruses in the causation of human cancers - a brief historical account. *Virology* 384: 260-265, 2009.
36. Haars R, Zentgraf H, Gateff E and Bautz FA: Evidence for endogenous reovirus-like particles in a tissue culture cell line from *Drosophila melanogaster*. *Virology* 101: 124-130, 1980.
37. Bell TM, Massie A, Ross MG, Simpson DI and Griffin E: Further isolations of reovirus type 3 from cases of Burkitt's lymphoma. *BMJ* 1: 1514-1517, 1966.
38. Kinzler KW and Vogelstein B: Cancer-susceptibility genes. Gatekeepers and caretakers. *Nature* 386: 761-763, 763, 1997.
39. Sottoriva A, Kang H, Ma Z, Graham TA, Salomon MP, Zhao J, Marjoram P, Siegmund K, Press MF, Shibata D, *et al*: A Big Bang model of human colorectal tumor growth. *Nat Genet* 47: 209-216, 2015.
40. Fojo T: Novel_target.com. *Oncologist* 6: 313-314, 2001.
41. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA Jr and Kinzler KW: Cancer Genome Landscapes. *Science* 339: 1546-1558, 2013.



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