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OPINION ARTICLE

Factors targeting *MED12* to drive tumorigenesis? [version 1; peer review: 3 approved with reservations]

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V1 First published: 22 Mar 2018, 7:359
<https://doi.org/10.12688/f1000research.14227.1>
 Latest published: 13 Dec 2018, 7:359
<https://doi.org/10.12688/f1000research.14227.2>

Abstract

Mediator Subcomplex 12 (MED12) is part of the transcriptional preinitiation machinery. Mutations of its gene predominantly occur in two types of highly frequent benign tumors, uterine leiomyomas and fibroadenomas of the breast, where they apparently act as driver mutations. Nevertheless, their presence is not restricted to benign tumors having been found at considerable frequencies in uterine leiomyosarcomas, malignant phyllodes tumors, and chronic lymphocytic leukemia also. Most of the mutations are located within exon 2 of the gene but in rare cases the intron 1/exon 2 boundary or exon 1 are affected. As to their type, predominantly single nucleotide exchanges with a hotspot in one codon are found, but small deletions clustering around that hotspot also are not uncommon. According to their presumed classification as gain-of-function mutations, these latter deletions are leaving the open reading frame intact. As to the types of mutations, so far no apparent differences between the tumor entities affected have emerged. Interestingly, this pattern with small deletions clustered around the hotspot of single nucleotide exchanges resembles that seen as a result of targeted gene editing. In contrast to other driver mutations the percentage of *MED12*-mutation positive tumors of independent clonal origin increases with the number of tumors per patient suggesting unknown etiological factors supporting site specific mutagenesis. These factors may act by inducing simultaneous site-specific double strand breaks the erroneous repair of which may lead to corresponding mutations. As inducers of DNA damage and its repair such as foreign nucleic acids of the microbiome displaying sequence homology to the putative target site might play a role. Interestingly, a 16 base pair homology of the hotspot to a putative terminator base-paired hairpin sequence of a *Staphylococcus aureus* tRNA gene cluster has been noted which might form R-loop like structures with its target sequence thus inducing said changes.

Keywords

Mediator subcomplex 12 (MED12), mutations, uterine leiomyomas, fibroadenomas of the breast, chronic lymphocytic leukemia (CLL), multiple tumors, DNA-RNA hybrids, *Staphylococcus aureus*

Open Peer Review

Approval Status

	1	2	3
version 2			
(revision)			
13 Dec 2018	view	view	
version 1			
22 Mar 2018	view	view	view

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Author roles: Bullerdiek J: Conceptualization, Formal Analysis, Writing – Original Draft Preparation, Writing – Review & Editing; Rommel B: Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: The author(s) declared that no grants were involved in supporting this work.

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How to cite this article: Bullerdiek J and Rommel B. **Factors targeting *MED12* to drive tumorigenesis? [version 1; peer review: 3 approved with reservations]** F1000Research 2018, 7:359 <https://doi.org/10.12688/f1000research.14227.1>

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Introduction

Surprisingly, the most common mutation in human tumors does not affect one of the famous suspects in the field (for review see (Vogelstein *et al.*, 2013)) but the much less well-known gene encoding Mediator Subcomplex 12 (*MED12*). *MED12* is part of the transcriptional preinitiation complex CDK8 (Elmlund *et al.*, 2006) and encoded by a gene that maps to the X-chromosome at Xq13.1. One obvious reason why its mutations so far have gained much less interest than those of other genes frequently mutated in human tumors is that they affect, to a large extent, benign tumors. Moreover, within malignant tumors, they are virtually absent from most of the predominant epithelial neoplasms like cancers of colon, breast, and lung (Kandoth *et al.*, 2013) whereas they only have been found at considerable frequencies in some malignant tumors of non-epithelial origin.

As in the benign tumors, however, mutations of that gene occur as apparent driver mutations in a predominant subset of uterine leiomyomas (Mäkinen *et al.*, 2011; Markowski *et al.*, 2012; McGuire *et al.*, 2012), constituting the by far most frequent human symptomatic tumors of all. Likewise, these mutations are also found in a large subset of fibroadenomas of the breast (Lim *et al.*, 2014; Piscuoglio *et al.*, 2015; Yoshida *et al.*, 2015), another frequent benign tumor which occurs predominantly in young and middle-aged women. Interestingly, they are not restricted to benign tumors but also frequently seen in their malignant counterparts, i.e. uterine leiomyosarcomas (Kämpjärvi *et al.*, 2012; Markowski *et al.*, 2013a; Pérot *et al.*, 2012) and malignant phyllodes tumors (Nagasawa *et al.*, 2015; Piscuoglio *et al.*, 2015; Yoshida *et al.*, 2015). Also, their presence in malignant tumors suggests that, albeit as a very rare event, certain additional mutations can trigger malignant transformation within formerly benign tumors harboring *MED12* mutations. Apart from solid tumors, *MED12* mutations recently were also detected in a significant percentage of roughly 5–9% of chronic lymphocytic leukemias (CLL) (Guièze *et al.*, 2015; Kämpjärvi *et al.*, 2015; Wu *et al.*, 2017a). Furthermore, the same type of *MED12* mutations was found in two canine vaginal leiomyomas (Markowski *et al.*, 2013a).

A closer look at the *MED12* mutations does not reveal apparent differences between the type of mutations when comparing the different tumor entities. Predominantly, the mutations are clustered in the 5' region of exon 2 of the gene with only a few mutations affecting the intron 1-exon 2 boundary or, much rarer, exon 1 or the exon 1-intron 1 boundary. Most of them are single base exchanges clearly clustered at two nucleotides of codon 44 where, albeit with different frequencies, guanins are found to be replaced by either A, C, or T. Besides these single base replacements, deletions and, more rarely, indels, usually affecting exon 2 or the intron 1/exon 2 boundary are found which always leave the reading frame intact indicating gain-of-function mutations. *MED12* maps to the X-chromosome and, as revealed by cDNA sequencing, the mutations are apparently restricted to the active X-chromosome (Mäkinen *et al.*, 2011; Markowski *et al.*, 2012; McGuire *et al.*, 2012).

We feel that for several reasons this highly frequent type of mutation might point to an unusual mechanism of mutagenesis

underlying the development of the corresponding tumors. These reasons will be discussed herein and a hypothesis based on target-specific mutagenesis will be presented. Starting with a short introduction of the main tumors affected by *MED12* mutations we will then address the molecular pathogenesis of uterine leiomyomas along with its clinical correlations followed by an in depth analysis of the pattern of *MED12* mutations. Finally, we will present a hypothesis why these data indicate a so far unknown etiological mechanism favoring these particular highly frequent somatic alterations of the genome.

Introducing three tumor entities displaying a unique type of *MED12* mutations

Besides a few very rare tumors, three main tumor entities are often affected by mutations of *MED12*. First, these three entities, i.e. uterine leiomyomas (fibroids), fibroadenomas of the breast, and chronic lymphocytic leukemias, will be introduced.

Uterine leiomyomas - the most frequent symptomatic human tumors

Uterine leiomyomas (UL) are benign smooth muscle tumors of myometrial origin with an apparently very low tendency to undergo malignant transformation. Depending on their location it can be distinguished between submucosal, intramural, and subserosal UL. Roughly 40–70% of women in their reproductive age will develop UL with a well-documented higher prevalence among women of African and African-American origin. In this group, the UL develop on average at younger ages (Laughlin *et al.*, 2010). Leiomyomas are often of large size and, as a matter of debate for more than 100 years, (Figure 1), multiple nodules occur at almost the same frequency or even more frequently than single nodules.

Nevertheless, the majority of patients with UL are without symptom. The remaining 20–30% of the patients suffer from symptoms like in particular heavy menstrual bleeding, increased menstrual periods, reduced fertility, and pelvic pressure and pain. During menopause, UL cease growing and even shrink. Nevertheless, despite their benign nature UL are the most symptomatic human tumors of all and repeatedly have been reported even in mummies with the oldest of them dating back to the Middle Neolithic age (3,200–2,500 BC) (Fornaciari & Giuffra, 2012). Surgery (hysterectomy or myomectomy) is a common type of treatment, but a variety of other non-surgical methodologies to treat UL are available (for review see: (Williams, 2017)).

While the etiology of these frequent tumors remains unclear (McWilliams & Chennathukuzhi, 2017), a bit more is known about pathogenetic factors. As to basic principles of their molecular pathogenesis, UL behave like the vast majority of other benign and malignant tumors. This includes a monoclonal origin which is triggered by so-called driver mutations. In the case of multiple UL almost every single nodule has been found to be of independent clonal origin (Mashal *et al.*, 1994). Accordingly, different nodules are usually characterized by different driver mutations in these cases.

Moreover, genetic subtypes, apparently belonging to different groups of driver mutations, exist. While *MED12* mutations,

Figure 1. Uterine leiomyomas in the scientific literature of the 19th century. Illustration of a uterus carrying multiple leiomyomas of various sizes and location (4, left) and uterus with a large pedunculated subserous leiomyoma (right, 6). Copperplate engraving from Virchow's series of lectures entitled "Die krankhaften Geschwülste" ("Morbid neoplasms") (Hirschwald, Berlin, 1864/65).

as a group, constitute the most predominant genetic subtype (Mäkinen *et al.*, 2011), another frequent subgroup of uterine leiomyomas carries rearrangements of the gene encoding the architectural transcription factor High Mobility AT-hook 2 (*HMG2*), as a rule reflected by cytogenetically visible chromosomal translocations (Schoenmakers *et al.*, 1995). Of note, the driver mutations of both subgroups occur in a mutually exclusive manner (Markowski *et al.*, 2012). Besides these subgroups, other, more rare but also independent genetic subgroups of UL such as one characterized by either germline (hereditary leiomyomatosis and renal cell cancer (HLRCC), OMIM 605839) (Bayley *et al.*, 2008) or somatic loss-of-function mutations of *Fumarate Dehydrogenase* (*FH*) seem to exist (Kämpjärvi *et al.*, 2016).

Fibroadenomas of the breast - Frequent benign tumors of adolescent and young women

Fibroadenomas of the breast are common benign tumors histologically composed of both stromal and epithelial components preferentially occurring in adolescent and young women. Their general incidence may be in the range of 10% in the corresponding age groups. Multiple tumors are not rare with some 10–15% of the patients having more than one FA.

Their name fibroadenomas (FA) and their classification as fibroepithelial tumors suggest a biphasic nature of these neoplasms. Nevertheless, as to their pathogenesis this classification seems to be misleading at least in the majority of cases because mutations are restricted to the stromal component of the tumors (Mishima *et al.*, 2015). Between 50% and 60% of the FA harbor *MED12* mutations (Lim *et al.*, 2014; Mishima *et al.*, 2015; Pfarr *et al.*, 2015). Histologically, the occurrence of *MED12* mutations correlates highly significant with the so-called intracanalicular growth pattern (Mishima *et al.*, 2015; Pfarr *et al.*, 2015). Interestingly, *MED12* mutations have also been found in considerable

percentages of other breast tumors of presumed stromal origin Phyllodes tumors and malignant Phyllodes tumors. In these tumors, the types of *MED12* mutations are not obviously discernible from those observed in UL and FA.

Chronic lymphocytic leukemias - most frequent leukemia of the adults

In Western countries, chronic lymphocytic leukemia (CLL) is the most common type of leukemia in adults. The American Cancer Society expects an estimated number of about 20,940 new cases of CLL in the United States in 2018 with about 4,510 CLL-related deaths. Overall, CLL accounts for about one-quarter of the new cases of leukemia. The average person's lifetime risk of getting CLL is about one in 175 (0.57%). The average age at the time of diagnosis is around 70 years, with men slightly more often affected than women. Frequently, the disease is detected in patients not yet showing any severe symptoms and in its early stages patients often undergo a 'watch and wait' period prior to starting therapy.

As to its pathogenesis CLL is a monoclonal leukemia of B-cell origin with a number of subsets that can be distinguished based on their genetic characterization, apparently pointing to different driver mutations. The genetic alterations also allow the stratification of groups which may require different therapeutic approaches. As a valid predictive parameter, deletions and mutations of the *TP53* gene are associated with a worse prognosis than other types of genetic changes and influence therapeutic decisions. Recently, mutations of *MED12* have been added to the list of potential driver mutations in CLL. Across the sub-types, they constitute a relatively rare genetic group affecting roughly 5–9% of CLL patients (Guièze *et al.*, 2015; Kämpjärvi *et al.*, 2015; Wu *et al.*, 2017a). Kämpjärvi *et al.* (Kämpjärvi *et al.*, 2015) have presented evidence that *MED12* mutations may represent a marker of worse prognosis.

A closer look at the molecular pathogenesis of uterine leiomyomas

According to the high prevalence of uterine leiomyomas *MED12* mutations are by far best investigated in this tumor type. Thus, we have now characterized the subset UL affected and described how they can be distinguished from other types of UL.

Leiomyomas with *MED12* mutation constitute their own genetic subtype which is also characterized by a distinct clinical and histopathological appearance

There is ample evidence that *HMGA2* rearrangements and *MED12* mutations occur mutually exclusively in UL and thus constitute independent driver mutations (Markowski *et al.*, 2012). Similarly, somatic *MED12* mutations and biallelic *Fumarate Hydratase* (*FH*) inactivation occur in mutually exclusive manner in both HLRCC syndrome-associated and sporadic uterine leiomyomas suggesting that the latter constitutes a third small group with an independent molecular pathogenesis (Kämpjärvi *et al.*, 2016). Accordingly, each of these genetic alterations alone as a driver mutation seems to be sufficient to induce the development of an UL without requiring any further mutations.

As to rearrangements of *HMGA2* and mutations of *MED12*, the transcriptome of tumors of both groups clearly differs with *MED12* mutation-positive and *HMGA2*-overexpressing samples clustering in distinct branches (Mehine *et al.*, 2013). Accordingly, both mutations allow the two major genetic subtypes of UL to be distinguished, and the question arises whether or not the genetic subtypes are also reflected by a different clinical behavior and histopathology.

Tumors carrying *HMGA2* rearrangements are usually solitary and, on average, of larger size than those with *MED12* mutations, also usually presenting as single tumors (Markowski *et al.*, 2014a) whereas the latter are smaller and often co-occur with other clonally independent nodules of the same genetic type

(Mäkinen *et al.*, 2011; Markowski *et al.*, 2012; Markowski *et al.*, 2014a). Among the women affected by *MED12*-mutation positive UL, more than two-thirds had more than one nodule (Heinonen *et al.*, 2017) (Figure 2A) of this type. Even more impressive, in the same study 52 (8.7%) *MED12*-mutation positive tumors made their appearance as the sole tumor with this mutation, while 547 (91.3%) tumors were associated with at least one other tumor of this genetic subgroup (Figure 2B).

To explain the high frequency of *MED12* mutated tumors among multiple leiomyomas, Heinonen *et al.* speculated that “the multiplicity of *MED12*-mutation-positive leiomyomas may derive from genetic predisposition and/or environmental factors rendering the myometrium susceptible to selection for *MED12* mutations” (Heinonen *et al.*, 2017). However, as outlined later herein, the association of multiple tumors with *MED12* mutations may be a key to the etiology of this type of UL.

Furthermore, *MED12*-mutated UL are also significantly associated with a subserous location compared to UL lacking this mutation (Heinonen *et al.*, 2017). As to histopathological features, a recent study by Wu *et al.* revealed that approximately 90% of the cells in *HMGA2*-rearranged UL were smooth muscle cells showing an overexpression of the protein, while in *MED12*-mutated UL a similar number of smooth muscle cells and other cells, i.e. mostly tumor-associated fibroblasts, were detected. These latter fibroblasts were lacking *MED12*-mutations (Wu *et al.*, 2017b) and thus apparently can be classified as by-stander cells. This fits with an earlier observation that in cell cultures of leiomyomas with *MED12*-mutations a rapid disappearance of mutated cells was seen that became replaced by UL wild-type cells, thus challenging the results of a variety of *in vitro* experiments on the biology of UL (Bloch *et al.*, 2017; Markowski *et al.*, 2014b).

Besides UL, the occurrence of *MED12* mutations has been well-documented in malignant uterine smooth muscle tumors (leiomyosarcomas) and smooth muscle tumors of uncertain malignant potential (STUMP), too (Holzmann *et al.*, 2015; Pérot *et al.*, 2012). Hence, an origin of these tumors from pre-existing UL has been suggested. In contrast, similar cases with *HMGA2* rearrangements have not been reported yet.

The percentage of *MED12*-mutated tumors is positively correlated with the total number of tumors per patient

To gain further insight into the biology of *MED12*-mutated UL, Heinonen *et al.* have undertaken a systematic attempt to check all feasible distinct tumors with a size of 1 cm or larger in diameter from hysterectomy uteri for *MED12* mutations (Heinonen *et al.*, 2017). In their study, 599 out of 763 leiomyomas carried *MED12* mutations (79%). Next, the data provided by the study of Heinonen *et al.* (Heinonen *et al.*, 2017) have been used to analyze the number of *MED12*-mutation positive UL per patient. While it was shown before that in the majority of patients having surgery *MED12*-mutated tumors do not make their appearance as single nodules but instead are accompanied by other yet clonally independent tumors of this same genetic type (cf. Figure 2), we were interested to see how the number of these tumors per patient is distributed in this series. A slow decrease of the number of

Figure 2. Single and multiple *MED12*-mutation positive uterine leiomyomas. Proportion of women carrying a single *MED12*-mutated UL (52/176) vs. those with more than one such tumor (124/176) (A) and proportion of *MED12*-mutated UL appearing as single tumor (52/599) vs. those accompanied by at least one other *MED12*-mutated UL (547/599) (B). Data according to Heinonen *et al.* (2017).

MED12-mutation positive tumors is noted (Figure 3). Nevertheless, from these Figures it is not possible to draw conclusions on the overall frequency and distribution of *MED12*-positive UL in the population because the results are biased by their restriction to symptomatic patients who had undergone surgical treatment. Nevertheless, they correspond more or less to the tumor numbers in general as seen from numerous other studies and thus the question arises if the *MED12*-positive tumors can be distinguished from the remaining UL as suggested by previous estimations (cf. Figure 2). Thus, we have next investigated if, in the case of multiple tumors, the percentage of *MED12*-mutation positive UL remains constant independent of the total number of tumors per patient. Surprisingly, it was noted that with a growing number of tumors, the percentage of *MED12*-mutated tumors clearly increased. Among solitary leiomyomas, only less than 40% of the tumors carried *MED12* mutations but their frequency was approaching nearly 100% if twelve or more tumors were present per patient (Figure 4). Thus, in contrast to other mutations, those of *MED12* seem to become more likely with an increase of the number of tumors.

Along with previous data this distribution confirms that, as for its pathogenesis, the occurrence of multiple leiomyomas near exclusively can be attributed to just one genetic mechanism, i.e. *MED12* mutations. This contradicts a statement by Mehine *et al.* that a shared clonal origin as a common feature of leiomyomas not carrying *MED12* mutations offers one explanation for the common occurrence of multiple concurrent lesions (Mehine *et al.*, 2015). Instead, a multitude of fibroids mainly appears to be a problem almost exclusively restricted to *MED12*-mutation

positive tumors and we thus decided to analyze and compare the different *MED12* mutations in more detail.

A closer look at the patterns of *MED12* mutations seen in various benign and malignant tumors

MED12 mutations occur in uterine smooth muscle tumors, fibroepithelial tumors of the breast and in chronic lymphocytic leukemia. As a rule, only subsets display these genetic abnormalities which range from the predominant alterations in UL to those detected only in a small percentage of cases in CLL. So far, there is no evidence that the tumors affected by *MED12* mutations in the three tumor entities described above differ with respect to their types of mutations. It has been speculated that unknown factors favor the occurrence of these particular mutations. To get further ideas on factors favoring them, the patterns of mutations have now been analyzed in more detail and compared between the different tumor entities paying particular attention to the small deletions occurring in exon 2 or at the intron1-exon 2 boundary, respectively.

In most cases single nucleotide exchanges are found with a clear predominance of those affecting nucleotides 130 and 131 belonging to codon 44. Less frequently other codons are mutated. Besides single nucleotide exchanges, deletions of small segments of the gene with varying sizes as well as indels affecting exon 2 or the intron1-exon 2 boundary are seen in some cases. As a rule, however, the transcript though affected by the deletions remains in frame. As to these latter genomic alterations accounting for roughly 15% of *MED12* mutations, we have analyzed the positions of the deleted bases from a variety of papers analyzing UL,

Figure 3. Number of *MED12*-mutation positive uterine leiomyomas per patient. Abscissa: number of UL/patient, ordinate: number of patients in the corresponding category. For this diagram data on *MED12* alterations published by Heinonen *et al.* (Heinonen *et al.*, 2017) have been used.

Figure 4. Number of uterine leiomyomas per patient and percentage of *MED12*-mutation positive tumors. Increasing percentage of *MED12*-mutation positive uterine leiomyomas (ordinate) with the number of tumors per patient (abscissa). Open rhombus indicates an interpolated value because no patients with 11 UL were present. For this diagram data on *MED12* alterations published by Heinonen *et al.* (2017) have been used.

fibroepithelial tumors, and CLL. Adding the number of deleted bases per each position reveals an almost symmetric distribution that is clustered around the hotspot of single nucleotide exchanges (Figure 5). In a previous study by our group, the beginning of the *MED12* deletions observed in uterine smooth muscle tumors was mostly located within exon 2 but in rare cases also upstream of the splice site within intron 1. Their size mainly ranged between 3 and 36 bp with a clear predominance of 15 and 18 bp (Markowski *et al.*, 2013b). Of note, an analysis of the data provided by Heinonen *et al.* for UL revealed that as very rare exceptions even larger deletions as well as those residing in exon 1 can occur.

As to size and position of these deletions, there is also no obvious difference between UL, fibroepithelial tumors of the breast, and CLL (Figure 5). Overall, this pattern of small deletions of various sizes clustered around the hotspot of single nucleotide exchanges resembles the results of genome editing based on targeted double-stranded breaks as for example those resulting from the usage of the CRISPR/Cas9 system (e.g. cf. Paquet *et al.*, 2016).

If these mutations indeed arise by certain types of repair of site-specific DNA changes, one might expect that many other mutations occur in the target region of *MED12*. Of these, only the “active ones”, i.e. those leaving the open reading frame intact and driving tumorigenesis, will lead to a clonal proliferation of their target cell giving rise to an UL whereas cells with other mutations of the hotspot region will remain quiescent or even become apoptotic (Figure 6). Therefore, future studies aimed at the detection of these “non-driving” mutations in single cells, especially from patients suffering from a multitude of UL, may be a

reasonable attempt. However, from the pattern of nucleotide exchanges and deletions, a commonly affected sequence can be depicted that may be related to the etiology of UL.

Hypothesis and opinion

MED12 mutations constitute highly frequent driver mutations in uterine leiomyomas and fibroadenomas, i.e. two tumor entities that occur almost exclusively in middle-aged and young women, respectively. In uterine leiomyomas, they even represent the by far most frequent genetic subtype with a clearly preferential occurrence in the case of multiple tumors. This is in sharp contrast to the other main genetic subtype of UL characterized by rearrangements of *HMG2* usually making its appearance in solitary nodules not accompanied by other tumors of the same genetic subtype.

It seems difficult to explain these findings just by independent random mutations followed by their selection. Nevertheless, additional factors favoring this multitude of tumors carrying the same type of mutations despite their independent clonal origin have remained enigmatic. After myomectomy, such factors may also account for the risk of recurrences that clearly increases with the number of UL that had been removed (Doridot *et al.*, 2001; Fauconnier *et al.*, 2000). Heinonen *et al.* (2017) have speculated that either genetic predisposition or environmental factors rendering the myometrium susceptible to selection for *MED12* mutations may contribute to the multiplicity of *MED12*-mutation positive tumors. To describe the development of multiple tumors, those two explanations are well-compatible with a model of clonally unrelated nodules that occur successively and are endowed with a different growth rate as depicted in Figure 7A. Another alternative explaining the

Figure 5. Patterns of *MED12* deletions in uterine leiomyomas, fibroepithelial breast tumors, and chronic lymphocytic leukemia. Left to right: Ideogram of the X-chromosome (commons.wikimedia.org), exon-intron structure of *MED12* (NCBI map viewer), and plot depicting frequency of deletions at each position around the preferred site of single nucleotide exchanges (red solid arrows) seen in uterine smooth muscle tumors (blue), fibroepithelial tumors of the breast (green), and chronic lymphocytic leukemia (yellow). Deletions are plotted across all deleted base positions. Minor preferred sites of single nucleotide exchanges within exon 2 are indicated by dashed red arrows. For this diagram data on *MED12* deletions from the following articles have been used: (Guièze *et al.*, 2015; Kämpjärvi *et al.*, 2015; Lim *et al.*, 2014; Markowski *et al.*, 2013b; Mishima *et al.*, 2015; Nagasawa *et al.*, 2015; Ng *et al.*, 2015; Pfarr *et al.*, 2015; Yoshida *et al.*, 2015) only those deletions beginning and ending in the displayed region have been considered.

multiplicity of UL is the occurrence of clonally related nodules with marked genetic evolution as shown by Mehine *et al.* (Mehine *et al.*, 2015) for UL lacking *MED12* mutations and depicted here as Figure 7B. Nevertheless, a variety of studies indicate that most tumors with *MED12* mutations are not clonally related and the multiplicity of these lesions thus needs other explanations. In addition to these both models possibly accounting for other genetic subtypes, as a third alternative factors as in particular infectious agents warrant consideration. The infection may lead to a synchronous initiation of multiple clonally independent lesions endowed with a different growth potential (Figure 7C).

As possible factors certain types of bacteria e.g. those involved in reproductive tract infections (RTIs) have long been suggested to be a cause of UL (e.g. (Witherspoon & Butler, 1934)). In the United States both reportable RTIs (i.e. chlamydia and gonorrhea) and fibroids disproportionately burden African American women which lead to the conclusion that the growth of fibroids might be triggered by inflammatory infections associated with the RTIs. Nevertheless, when exploring the relationship between self-reported RTIs and fibroid size, number, and total volume Moore

Figure 6. *MED12* mutations in uterine smooth muscle tissue. Model illustrating the occurrence and selection of *MED12* mutations during the course of leiomyoma development. The scheme suggests that of a larger number of *MED12*-mutations only those associated with a gain of function act as driver mutations giving rise to UL.

et al. did not find strong associations (Moore *et al.*, 2015). In a recent contribution by the same group women seropositive for genital *Chlamydia trachomatis* were even found to be less likely to have fibroids (Moore *et al.*, 2018). In line with these findings, in the study by Heinonen *et al.* neither a history of pelvic inflammatory disease (PID) nor of *Chlamydia* infection was found to be significantly associated with the *MED12*-type UL while PID turned out to be significantly associated with the occurrence of *MED12*-wild type UL (p 0.0024) (Heinonen *et al.*, 2017).

Akin to bacteria, viruses have also been suggested to be involved in the development of UL. For example, *EBV* is known as a factor associated with the development of extra uterine smooth muscle tumors in HIV and post-transplant patients (see e.g. (Miettinen, 2014; Purgina *et al.*, 2011; Ramdial *et al.*, 2011)). However, so far no association between *EBV* and uterine leiomyomas has been demonstrated. As to another virus of the Herpes group, a recent study failed to reveal a significant association between *HSV-2* seropositivity and the presence of fibroids (Moore *et al.*, 2016) and in general no convincing evidence for viruses involved in the pathogenesis of UL has been presented.

While infectious diseases as etiological agents of UL repeatedly have been assumed as such the question arises as to how they could act by site-specific targeting the hotspot region of mutations residing within exon 2 of *MED12* and which infectious agents are possible candidates. We will herein present the opinion that the interaction between the human DNA and foreign nucleic acids derived from the infection plays a causal role. As an unorthodox hypothesis stimulating further discussions, we would like to advance the hypothesis that the *MED12* mutations result from cleavage of R-loop structures. By definition, R-loops are derived from double stranded DNA where one strand forms a stable DNA-RNA hybrid helix whereas the former associated DNA strand remains

Figure 7. Different models of time and clonality of uterine leiomyoma development. Three alternative models explaining the development of multiple uterine leiomyomas are depicted: (A) corresponds to a *prima facie* model of myomagenesis with multiple independently developing nodules having a different growth rate. (B) Illustrates the development of multiple clonally related tumors from one common predecessor as described by Mehine *et al.* (Mehine *et al.*, 2015) for *MED12*-mutation negative UL. (C) offers an alternative explanation for the growth of multiple clonally unrelated nodules with simultaneous initiation but a different growth rate. Open circles indicate single mutated cells of origin affected by driver mutation.

single-stranded. R-loops with an "exposed" stretch of single-stranded DNA can give rise to instability and DNA double-strand breaks (Aguilera & Gómez-González, 2017; Freudenreich, 2018; Su & Freudenreich, 2017). While the hybrid helix is usually composed of DNA with endogenous RNAs it seems possible that such helices can be formed with foreign RNA as well. To this end, it has been hypothesized that circulating exogenous RNA sequences after their uptake may influence the function of cells through miRNA-like mechanisms (Wang *et al.*, 2012) suggesting direct influences of these sequences of the microbiome on its host's cells.

To search for possible sequence homologies we have depicted a target region 5'TGTAAACAAGGTTTCAATAAC3' covering 10 nucleotides upstream and downstream each of the two c. 130 and c. 131 (GG), respectively (cf. Figure 6). Of the resulting list with at least 15 identical nucleotides, a variety of human pathogens have been identified as e.g. *Bacteroides fragilis* (nt 2-20), *Klebsiella pneumoniae* (nt 2-20), *Escherichia coli* (nt 3-20), *Vibrio vulnificus* (nt 6-22), *Staphylococcus aureus* (nt 6-22, and nt 1-16, respectively), *Staphylococcus argenteus* (nt 6-22), and *Clostridium botulinum* (nt 3-19). When searching for abundantly expressed sequences an interesting candidate emerged. A 16-base pair sequence identical to the sense strand of the sequence of a *Staphylococcus aureus* 27-tRNA gene cluster immediately 3' to an rRNA

operon (Green & Vold, 1993) was noted. The homology covers a palindromic sequence which may act as a terminator of transcription (Green & Vold, 1993) and may also lead to the formation of a hairpin structure stabilizing the RNA molecule (Figure 8). Are these molecules likely to exist as circulating RNAs? *Staphylococcus aureus* belongs to the phylum of *Firmicutes* in general constituting the third most abundant sequence population in human plasma with a significant number of the reads mapping to various bacterial ribosomal RNAs and tRNAs (Wang *et al.*, 2012). More specifically, evidence has been presented that RNA species from *Staphylococcus* are commonly present in blood (Leung & Wu, 2015).

In summary, deduced from the types and patterns of *MED12* mutations in human tumors we have presented evidence supporting the idea that nucleic acid sequences of the human microbiome may interact with the common hotspot of *MED12* mutations. To stimulate further discussion a possible interaction of a sequence of *Staphylococcus aureus* with this hotspot has been considered in more detail as depicted in Figure 8 and Figure 9. As to the initial stage of leiomyoma development, the clearance of R-loops resulting from a hybrid helix between a human target cell and bacterial RNA may simultaneously give rise to multiple clonally independent cells of origin. Additional factors such as the site of origin,

Figure 8. Sequence adjacent to a 27-tRNA gene cluster of *Staphylococcus aureus* similar to the *MED12* hotspot. Upper part: Homology of the human *MED12* hotspot region (red arrow) with the sense strand of the sequence of the *Staphylococcus aureus* 27-tRNA gene cluster immediately 3' to a rRNA operon (Green & Vold, 1993). Blue dashed arrows indicate a palindromic sequence. Sequence from *Staphylococcus aureus* strain CFSAN007847 chromosome, complete genome; GenBank: CP017684.1; GenBank: FASTA; NCBI Blast. Lower part: bacterial tRNA and rRNA genes of the operon adjacent to the site of homology are shown in blue.

Figure 9. Model depicting the interaction between bacterial RNA with the *MED12* mutational hotspot. As an example for the putative sequence of the human microbiome inducing site specific mutations this scheme refers to Figure 8.

angiogenetic support, and the type of *MED12* mutation, may endow the resulting monoclonal lesions with a different growth potential.

Data availability

All data underlying the results are available as part of the article and no additional source data are required.

Competing interests

No competing interests were disclosed.

Grant information

The author declared that no grants were involved in supporting this work.

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Reviewer Report 31 July 2018

<https://doi.org/10.5256/f1000research.15482.r36051>

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Jose M. Teixeira

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The opinion article by Bullerdiek and Rommel provides an interesting hypothesis that is partly supported by the unusual role of MED12 in the origin of uterine leiomyoma. MED12 is mutated in these benign tumors at a hotspot location at the 5' end of exon 2. How these mutations arise in the myometrium and how the mutant MED12 drives leiomyoma development are not known.

I agree with many of the previous reviewers' comments and will limit my comments to a few items so as not to be too repetitious. For example, I agree that there is no evidence that the MED12 mutations constitute a gain-of-function in the protein. Indeed, if MED12 mutation alone is sufficient for tumorigenesis, it would have been observed in a variety of other tumor types. In fact, no mechanisms for tumorigenesis unique to leiomyomas have been reported. Thus, how MED12 mutation leads to leiomyoma development is not known at this point. This should be changed in the text.

I also agree that the argument for foreign RNA from bacterial infection causing MED12 mutation, although quite novel and certainly interesting, is weak. In addition to the reasons already discussed, ascending infections, such as the reproductive tract infections described by the authors, are likely to rarely involve the myometrium. In the case of *S. aureus*, it is even more unlikely because myometrial infection with that organism is likely associated with bacteremia, which is even more rare and cannot account for the prevalence of MED12 mutant fibroids in women. Also, although leiomyomas are normally found in reproductive age women, that doesn't necessarily mean that sexually active women are more prone to the disease because of possible sexually transmission of pathogenic bacteria. Since parity is associated with a lower leiomyoma burden, the argument for reproductive tract infections being the culprit is not supported. Parity is also associated with increased risk of postpartum iatrogenic infection, but again parity is associated with decreased risk for leiomyomas. These caveats should be included in the text.

There is something special about the very common MED12 mutations in that hotspot and high

prevalence of uterine leiomyomas (and the not-so-common fibroadenomas) in reproductive age women. It is possible that a hormonally-regulated factor expressed only in myometrium (and breast stroma) could be interacting with wt MED12 on the site where the hotspot mutations alter the protein structure, and mutation disrupts that interaction. How those MED12 mutations occur and why the tumors/fibroadenomas develop only in those tissues is a mystery that needs to be resolved in order to develop therapies targeting the mechanisms involved.

Is the topic of the opinion article discussed accurately in the context of the current literature?

Partly

Are all factual statements correct and adequately supported by citations?

Partly

Are arguments sufficiently supported by evidence from the published literature?

Partly

Are the conclusions drawn balanced and justified on the basis of the presented arguments?

No

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 19 June 2018

<https://doi.org/10.5256/f1000research.15482.r34711>

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Eric Glasgow

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This opinion article by Bullerdiak *et al.* reviews the types and etiologies of tumors associated with MED12, and put forward a hypothesis regarding the mechanism of MED12 mutation. MED12-associated tumors are understudied considering their high frequency, probably because they are most commonly associated with benign tumors. However, a better understanding of the etiology of these tumors would likely contribute substantially to our knowledge of the genetic mechanisms of tumorigenesis. The authors discuss the main types of tumors caused by MED12 mutations: uterine leiomyomas (ULs), fibroadenomas of the breast, and chronic lymphocytic leukemias. They point out that the characteristics of ULs associated with MED12 mutations differ markedly from those of ULs with other genetic causes. Within the same patient, multiple tumors with MED12

mutations of independent clonal origin are common. Bullerdiel *et al.* illustrate the presence of a mutational hotspot within the *MED12* sequence with homology to tRNAs of multiple human pathogens. These qualities are suggestive of a genome editing mechanism warranting further investigation.

Important changes:

- The gene name! The gene name is mediator complex subunit 12 (MED12), not Mediator Subcomplex 12 as stated in the article.

- In the Abstract it is stated "(MED12)...presumed classification as gain-of-function mutations...", and in the Introduction "...deletions and, more rarely, indels, usually affecting exon 2 or the intron 1/exon 2 boundary are found which always leave the reading frame intact indicating gain-of-function mutations". The term gain-of-function should be removed. The function of MED12 is unknown and there is no evidence suggesting that these mutations are gain-of-function.

Major issues:

- In Figure 2B, it is unclear how many patients in total were analyzed for tumor counts or whether the multiple *MED12*-mutation positive UL were concentrated in a small number of patients or spread evenly across patients analyzed.

- Additionally, the labels appear to be switched in the key of Figure 2.

- A major problem is that the authors claim that the occurrence of multiple ULs can be exclusively attributed to *MED12* mutation. However, although the authors provide ample quantitative evidence of multiple ULs resulting from *MED12* mutation within the same patient, the authors show no data regarding the occurrence of multiple ULs with other genetic mechanisms (i.e. *HMG2* arrangements). In order to make the claim that multiple UL is a feature unique to *MED12*-mutated tumors, the authors should provide data on non-*MED12*-mutated multiple UL for the sake of comparison. If the number of non-*MED12*-mutated multiple UL is zero, this should be made clear within the text of the article.

- In reference to Figure 5, it is unclear whether the CLL mutations (yellow) cluster around the mutational hotspot as do the UL and fibroadenoma mutations, although the text of the article claims that the CLL mutations also cluster around the hotspot.

- The authors point out that the evidence for a connection between infection and UL is dubious thus far, yet they go on to propose a mechanism for the role of infection in UL pathogenesis. To make a convincing argument, the authors must clarify why they still consider a pathogenic mechanism worth considering.

- The authors should provide more detail about the pathogens with tRNA sequences matching the mutational hotspot. What is known about the involvement of these pathogens in pelvic infections? Does the prevalence of pelvic infections involving these pathogens approximately match the prevalence of UL? Is there any existing evidence for association between these pathogens and UL?

Minor issues:

- Second paragraph of the introduction: "As in the benign tumors, however, mutations of that gene

occur as apparent driver mutations in a predominant subset of uterine leiomyomas (Mäkinen *et al.*, 2011; Markowski *et al.*, 2012; McGuire *et al.*, 2012), constituting the by far most frequent human symptomatic tumors of all." Do the authors mean "As in the malignant tumors..." Also, "that gene" is vague. Replace with *MED12*.

-Second paragraph introduction: "Furthermore, the same type of *MED12* mutations was found in two canine vaginal leiomyomas (Markowski *et al.*, 2013a)" can be changed to "Furthermore, the same type of *MED12* mutation was found in two canine vaginal leiomyomas (Markowski *et al.*, 2013a)"

-Third paragraph introduction: "Predominantly, the mutations are clustered in the 5' region of exon 2 of the gene with only a few mutations affecting the intron 1-exon 2 boundary or, much rarer, exon 1 or the exon 1-intron 1 boundary" can be changed to: "Predominantly, the mutations are clustered in the 5' region of exon 2 of the gene with only a few mutations affecting the intron 1-exon 2 boundary or, more rarely, exon 1 or the exon 1-intron 1 boundary"

-The heading "Introducing three tumor entities displaying a unique type of *MED12* mutations" should be changed to "Introducing three tumor entities displaying *MED12* mutations"

-Under subheading: Uterine Leiomyomas – the most frequent symptomatic human tumors, "Depending on their location it can be distinguished between submucosal, intramural, and subserosal UL" can be changed to "Depending on the location, the difference between submucosal, intramural, and subserosal UL can be distinguished"

-The subheading "Fibroadenomas of the breast - Frequent benign tumors of adolescent and young women" can be changed to "Fibroadenomas of the breast - Frequent benign tumors in adolescent and young women" and the subheading "Chronic lymphocytic leukemias - most frequent leukemia of the adults" can be changed to "Chronic lymphocytic leukemias - most frequent leukemia in adults"

-Under the heading "A closer look at the molecular pathogenesis of uterine leiomyomas," the sentence, "According to the high prevalence of uterine leiomyomas *MED12* mutations are by far best investigated in this tumor type" can be changed to "*MED12* mutations are by far best investigated uterine leiomyoma tumor type."

-Second paragraph under the subheading: "Leiomyomas with *MED12* mutation constitute their own genetic subtype which is also characterized by a distinct clinical and histopathological appearance," the sentence "Accordingly, both mutations allow the two major genetic subtypes of UL to be distinguished, and the question arises whether or not the genetic subtypes are also reflected by a different clinical behavior and histopathology" can be changed to "Accordingly, these mutations allow the two major genetic subtypes of UL to be distinguished, and the question arises whether or not the genetic subtypes are also have different clinical behaviors and histopathologies"

-The second paragraph under the subheading: "The percentage of *MED12*-mutated tumors is positively correlated with the total number of tumors per patient," the sentence "Along with previous data this distribution confirms that, as for its pathogenesis, the occurrence of multiple leiomyomas near exclusively can be attributed to just one genetic mechanism, i.e. *MED12*

mutations" can be changed to: "Along with previous data this distribution confirms that the occurrence of multiple leiomyomas nearby can be exclusively attributed to just one genetic mechanism, i.e. *MED12* mutations."

-At the end of the first paragraph under the heading: "A closer look at the patterns of *MED12* mutations seen in various benign and malignant tumors" the word respectively is unnecessary.

-Under "Hypothesis and Opinion" the sentence "Nevertheless, additional factors favoring this multitude of tumors carrying the same type of mutations despite their independent clonal origin have remained enigmatic" should be changed to "Nevertheless, additional factors favoring this multitude of tumors with independent clonal origin carrying the same type of mutation have remained enigmatic."

-Also, "In addition to these both models possibly accounting for other genetic subtypes, as a third alternative factors as in particular infectious agents warrant consideration" should be changed to "In addition to these models, the potential roles of infectious agents warrant consideration"

-Also, "As to another virus of the Herpes group, a recent study failed to reveal a significant association between *HSV-2* seropositivity and the presence of fibroids (Moore *et al.*, 2016) and in general not convincing evidence for viruses involved in the pathogenesis of UL has been presented" should be changed to "As to another virus of the Herpes group, a recent study failed to reveal a significant association between *HSV-2* seropositivity and the presence of fibroids (Moore *et al.*, 2016) and in general no convincing evidence for involvement of viruses in the pathogenesis of UL has been presented"

To summarize, the authors build a fairly convincing argument for the need to more closely study the mechanism of *MED12* mutation in tumorigenesis. However, as detailed above, there are multiple areas in which additional evidence and clarification is needed to support the authors' claims. Additionally, correction of several grammatical errors and awkward phrasing throughout the manuscript would greatly improve readability.

Is the topic of the opinion article discussed accurately in the context of the current literature?

Partly

Are all factual statements correct and adequately supported by citations?

Partly

Are arguments sufficiently supported by evidence from the published literature?

Partly

Are the conclusions drawn balanced and justified on the basis of the presented arguments?

Partly

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 30 April 2018

<https://doi.org/10.5256/f1000research.15482.r33313>

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Takeshi Kurita

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This Opinion Article proposes a provocative idea that transcripts of *Staphylococcus aureus* contribute to an exceptionally high incidence of *MED12* exon 2 mutations among human tumors. After providing an excellent summation of the current literature on demographics and characteristics of *MED12* mutations in human neoplasms, the authors propose an intriguing concept: the involvement of mutagenic nucleic acids of bacterial origin in human tumors, based on the discovery of a *Staphylococcus* genome sequence of which the transcript may form an R-loop with human *MED12* gene on the mutation hotspot. This novel concept merits further discussion among researchers of diverse biomedical fields.

There are several issues that I recommend the authors to address.

I. The importance of the idea would increase if the following issues are resolved.

- The methods of database search for homologous sequences and the statistical significance of the finding should be described. In other words, it is unclear how frequently such homologous sequences to the human genome appear in the genome of pathogenic microorganisms, and what proportion of such homologous sequences can form an R-loop with human genome if they are transcribed.
- The palindromic sequence is located at 30 bp from the 3' end of tRNA-Leu on the reverse strand in *Staphylococcus aureus* genome. Since it is not a typical template for transcription, inclusion of references that suggest transcriptional activity in such genomic regions of bacteria would help further discussion by increasing the feasibility of the proposed model.
- It would also be helpful to include a discussion on the possible mechanisms through which naked transcripts of low-copy number in the circulation could possibly reach the target locus in the genome of myometrial cells. For instance, internalization of shRNA for RNAi is achieved by viral transduction or chemical/physical transfection of shRNA expression vectors.
- The authors may speculate the mechanisms that unfold the minimum free energy structure of short hairpin transcripts and facilitate the hybridization to the genomic DNA. For example, shRNA is processed into siRNA, and then siRNA forms a complex with cellular proteins to elicit RNAi effect.

II. There are issues in data presentation

- Regarding the title of Figure 2: "Single and multiple *MED12*-mutation positive uterine leiomyomas", the adjectives "single and multiple" could modify "*MED12*-mutation". Adding a hyphen (*MED12*-mutation-positive) and using of "solitary" instead of "single" would improve the readability.

- Error: The labels for single and multiple UL groups in Figure 2 are switched.
- The graphs in Figures 2, 3 and 4 are the same data in different presentation format. The Figures 3 and 4 seem to be redundant.

III. There are other factors that also likely contribute to the high prevalence of patients with multiple *MED12* mutant ULs.

1. A single hit on the active *MED12* allele is sufficient for the pathogenesis of ULs. Human cancer cells usually carry multiple putative driver mutations even at the earliest stage. In contrast, most *MED12* mutant ULs do not carry additional mutations, suggesting a *MED12* mutation is sufficient to drive UL pathogenesis. Since the *MED12* is on X-chromosome, a single hit on the active allele of *MED12* has dominant effect. Hence, the development of UL through *MED12* mutations should occur at a significantly higher rate compared to the neoplasms that require multiple genetic lesions for pathogenesis.
2. Diversity of pathogenic *MED12* mutations increases the prevalence of *MED12*-mutant ULs. Generally, driver missense mutations of human neoplasms are very specific. For instance, nearly all adult-type granulosa cell tumors carry *FOXL2* c.402C>G (C134W) missense mutation. While single base replacements in the hotspot triplet bases can also result in conversion of C134 to F, S, Y, R and G, only C134W is pathogenic. In contrast, a variety of *MED12* mutations, ≥ 10 missense and > 30 indel mutations, are associated with ULs. For instance, missense mutations that convert *MED12* G44 to D, S, V, R, C and A are all pathogenic. Hence, even if the mutation rate per nucleotide is equal throughout the human genome, the incidence of pathogenic *MED12* mutations should be many times higher than other pathogenic mutations.
3. UL is a hormone dependent tumor. Another key factor that this Opinion Article does not address is the systemic hormonal environment. The pathogenesis of UL depends on estrogen and progesterone. Since ULs are counted only when they grow to a grossly recognizable size, patients with endocrine profiles favorable to the growth of ULs should have a higher number of tumors even if the incidence of pathogenic mutations in myometrial cells is equal among all women.

Discussion of these factors is not essential, as the model proposed in this article would work independently. Nevertheless, these additions would help balance the discussion.

Is the topic of the opinion article discussed accurately in the context of the current literature?

Yes

Are all factual statements correct and adequately supported by citations?

Yes

Are arguments sufficiently supported by evidence from the published literature?

Partly

Are the conclusions drawn balanced and justified on the basis of the presented arguments?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biology of Uterine Leiomyoma

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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