"Characterization of the Pathogenicity of the MSH2 P640T Mutation in Saccharomyces cerevisiae"

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Excerpt

Discussion

Summary: The results obtained in this paper provide a model for the pathogenicity of the P640T mutation in yeast MSH2, the human homolog of which has previously been linked to HNPCC (Gammie et al. 2007; Leach et al. 1993; Chialina et al. 2006). We are further interested in studying this mutation due to the significantly increased mutation rates found to result from it by previous studies by ourselves and others (Lang et al. 2013; Arlow et al. 2013). Here, the mutation was found to lead to decreased nuclear localization (1.05±.025 vs. 1.26±.019) via an immunofluorescence assay, decreased steady state levels of the MSH2 protein to the point of the protein being absent (.97% of the wildtype steady state levels) via an immunoblot analysis, and decreased interactions between MSH2 and its functional binding partners in a yeast 2-hybrid assay.

msh2-P640T has decreased nuclear localization: In order for MSH2 to function correctly, it must localize to the nucleus (Kunkel & Erie 2005; Modrich 2006). In order to test for the possibility of mislocalization of MSH2 being the cause of the mutation's previously observed pathogenicity, we conducted an immunofluorescence assay, allowing us to determine the localization of the MSH2-P640T protein.

We found that the wildtype MSH2 has a nuclear concentration that is 1.25±.019 times that of the cytoplasm, which we take to be the normal wildtype localization ratio. MSH2-P640T, on the other hand, has a significantly lower nuclear-to-cytoplasmic ratio of 1.05±.025 ($p = 1.8x10\text{-}14$) (Fig 3b). A ratio this near 1 could result from either of two possibilities: either the protein is present in equal concentrations in both the nucleus and the cytoplasm, or it is equally absent in both parts of the cell and our recordings are of background fluorescence. In the context of the results of our second experiment, the immunoblot, which demonstrates that there is no protein in the cell, it can be concluded that the 1.05±.025 N/C of P640T results from the MSH2 protein being absent in both the nucleus and the cytoplasm. This is supported by the finding that the null strain lacking MSH2 completely has a similar nuclear-to-cytoplasmic ratio of 1.10±.006, with no statistically significant difference from the P640T strain's ratio ($p = .057$) (Fig. 3b). Here, there is no MSH2 to localize, so any fluorescence readings that cause a deviation from a N/C of 1 are due only to background fluorescence. Overall, msh2-P640T's decrease in nuclear localization is likely due to the fact that there is no protein, as demonstrated by another of our experiments, the immunoblot.

msh2-P640T is absent from the cell under normal expression conditions: Increased mutation rates resulting from a lack of MSH2 activity in MMR could also be due to the protein being absent, which may result from a missense mutation (Gammie et al. 2007). Indeed, previous studies of missense mutations in the MSH2 protein have found that certain mutations,

including P640T, result in lower MSH2 levels overall (Arlow et al. 2013; Gammie et al. 2007). In order to determine if the increased mutation rates of msh2-P640T are due to decreased levels of the protein, we conducted an immunoblot analysis.

The data obtained from our immunoblot indicate that the mutated MSH2 protein is expressed at a significantly lower level than in the wildtype and is absent from the cell, so low that the protein is essentially absent from the cell. Specifically, we found that the P640T strain expressed MSH2 (found at the expected 105 kDa location) at .97% the level of the wildtype strain, indicating that it is unstable and is essentially not present in the cell. Although the null strain was calculated to have a .86% expression level of MSH2 compared to the wildtype when it was expected to have no MSH2 expression, this expression level is negligibly low. The similarity between the steady state levels of the MSH2-P640T strain and the null strain demonstrates the substantial level of decreased MSH2 protein. Such a significant decrease in functional MSH2 provides an explanation for the increased mutation rates observed in previous studies, for if there is no MSH2 to participate in MMR, mutations will accumulate (Lutzen et al. 2000; Gammie et al. 2007). Our findings are supported by similar findings of decreased steady state levels in other studies of P640T and similar mutations (Gammie et al. 2007; Arlow et al. 2013).

In light of these findings, we posit that the decreased steady state levels are the product of protein instability and subsequent degradation of the mutated protein, due to the fact that our mutation was not located in a transcriptionally relevant portion of the DNA sequence (i.e., the promoter). Therefore, the protein is likely to be transcribed and translated normally by the cell, but to be structurally destabilized by the mutation and therefore degraded by the cell, leading to the decreased steady state levels observed in the immunoblot. Such degradation of unstable proteins is not uncommon and has been found to occur to missense mutated MSH2 in other studies, including MSH2-P640T (Lutzen et al. 2000; Gammie et al. 2007; Arlow et al. 2013). This model is supported and better elucidated with a structural analysis of the MSH2 protein.

Structural Analysis of the P640T mutation indicates that the Pro640 residue is structurally significant: Using human MutS as the model and information from previous studies, we identified the human homolog of the Pro640 residue to be part of a highly evolutionarily conserved "stability cluster" that stabilizes both interdomain interactions and the dimer interface (Obmolova et al. 2000; Gammie et al. 2007) (Fig. 5). Additionally, mutations in this highly conserved region have previously been found to cause pathogenic phenotypes similar to those observed to result from the P640T mutation in yeast MSH2 (Gammie et al. 2007). Furthermore, the residue is located in MSH2's ATPase domain, whose function is known to be vital for successful MMR (Alani et al. 1997).

These findings lead us to believe that, because the Pro640 residue is important in maintaining the structure of MSH2 (Obmolova et al. 2000; Gammie et al. 2007), the P640T mutation severely alters the conformation of the protein, compromising its stability and leading to degradation by the cell.

Yeast 2-hybrid assay confounded by possible degradation of MSH2: Because MMR is largely dependent on the correct functioning and interaction of MSH2 with its functional binding partners (Kunkel & Erie 2005; Chang et al. 2000; Guerette et al. 1999), and because single amino acid changes have the capability of disrupting binding partner interactions (Gammie et al. 2007), we conducted a yeast 2-hybrid assay to determine whether MSH2-P640T is still capable of interacting with its binding partners. Notably, the yeast 2-hybrid system is an

overexpression system, which would ideally allow for observation of P640T's interaction ability despite its almost nonexistent steady state level under normal expression conditions.

We found the MSH2-P640T protein to lack interaction with any of the studied functional binding partners (Fig. 3b). In the context of the earlier described structural model, there are two possible explanations for these findings: either a sufficient amount of protein is present in the cell but no interactions occur or, as seen in the immunoblot, MSH2 is still not present in the cell despite overexpression, and therefore no interactions can occur with it. First, MSH2-P640T's inability to interact with its binding partners could be due to destabilization of the protein's ability to interact with its partners. Without the correct structure, MSH2 may not be able to interact properly with other MMR proteins, preventing MMR from proceeding correctly and leading to increased mutation rates.

A second explanation for the lack of observed interaction follows from the findings of the immunoblot. In particular, the effect of the P640T mutation on MSH2's stability could be so destabilizing that even under over expression conditions, the protein is still degraded before it can interact with any of its binding partners (Lützen et al. 2008). This is supported by the known role of Pro640 in maintaining domain-domain interactions and the protein's structure as a whole (Gammie et al. 2007; Obmolova et al. 2000), suggesting that mutating the residue leads to destabilization of such domain-domain interactions and of MSH2 altogether. In this scenario, no interactions would be observed in a yeast 2-hybrid assay, since the MSH2 protein is not present for interaction. Such a lack of interactions has been determined to be at the root of the pathogenicity of other MSH2 mutations, including C67Y, S762Y, and L457P (Gammie et al.,= 2007).

In order to determine whether the lack of interaction observed in the yeast 2-hybrid assay is due to a misfolded MSH2 or a nonexistent MSH2, we suggest that a yeast 2-hybrid assay with overexpression be performed simultaneously with an immunoblot with overexpression to determine whether the overexpression allows for a significant amount of MSH2 to remain in the cell to interact with other proteins. If MSH2 were observed to be present under overexpression conditions, and similarly lacking interactions were observed in the yeast 2-hybrid assay, then it can be concluded that the MSH2 protein fails to interact with its binding partners due to its being misfolded. However, if it were observed that MSH2 continues to be absent in the immunoblot despite being overexpressed, it can be concluded that the yeast 2-hybrid assay does not accurately reflect protein-protein interactions.

Destabilization and degradation model: We propose a model to explain the pathogenicity of the P640T mutation. In light of the position of Pro640, which has previously been determined to play an important role in maintaining interdomain interactions, interactions between binding partners, and the structural stability of the protein as a whole (Gammie et al. 2007; Obmolova et al. 2000; Lang et al. 2013), we hypothesize that mutating the residue causes a major structural destabilization, which causes the protein to be degraded by the cell before it can localize to the nucleus. Such instability and degradation were first indicated in our immunoblot, as MSH2-P640T levels were found to be nonexistent.

Moreover, this is in accordance with our immunofluorescence assay results, which displayed no MSH2 in the cell, presumably a result of the protein being degraded. Our model also provides a possible reason for the lack of interaction seen in the yeast 2-hybrid assay: degradation by the cell. With such a significant drop in MSH2 steady state levels as that seen in the immunoblot, we postulate that the level of degradation of MSH2 is such that, even in an overexpression system, there is still not enough protein remaining after degradation to interact

with other proteins. Such degradation is not uncommon for the mutation of a structurally relevant proline, as studies of other proteins, including MSH2, have observed similar results (Arlow et al. 2013; Andre & Pereira 2008; Schultz et al. 2005).

Author Commentary

Ramie Fathy

Writing the Discussion was easily the most satisfying part of this assignment. Unlike in literary writing, where the writer leads the reader to a conclusion that reinforces the thesis at the end of every argument, in scientific writing the real analysis of the results is not done until the Discussion section. This made for a very frustrating attempt to make sure I only outlined the findings in the Results section, without analyzing it until the next section. However, this frustration quickly dissipated upon beginning my analysis in the Discussion section. Ultimately, saving the analysis until the Discussion section helped me craft a better interpretation of the findings, as it forced me to take all of the results into consideration before pursuing any possible model of the results based on the findings of one or two of the experiments. Outlining all of the data and all of the findings helped to organize the information in my head as well, allowing me to better piece it all together into a model that accurately accounts for all of the results.

I used this to reorganize my Discussion into an order that introduced the most relevant findings to the reader first, providing a basis that the reader can use to analyze the rest of the findings and to make sense of why certain experiments were no longer relevant. For example, I first discuss the results of the localization and expression assays, which demonstrated that the MSH2 protein was neither present nor expressed in the cell. I then use this information as a motivation for studying the destabilizing effects of the P640T mutation on the structure of the MSH2 protein, revealing that the amino acid residue is located in a vital location for the protein's stability, providing an explanation for the decreased steady state levels of the protein. Importantly, this interpretation brings into question the results of the yeast 2-hybrid assay, a confounding that I then discuss in the paper. In addition, I provide possible experiments to clarify this confound and test my interpretation of the results. Here, I have reorganized my discussion of the experiments and their significance from the original order that I performed them, in order to first provide the reason for an experiment's failure or findings before discussing the results. Unlike the Results section, where it was tantalizing to avoid analysis, the Discussion section was exciting to write, as I was finally getting my chance to bring the reader to a satisfyingly explanatory model.

Importantly, I succinctly reiterate all of the relevant background information before analyzing the results of a specific experiment or set of experiments. Doing so enabled me to frame the findings so that the reader interprets them in the context of a specific set of background facts and does so with a better understanding of the process and protein in question. Writing these mini-introductions was easier than writing the actual introductions, since my motivation for including these facts is more limited to each experiment than by the entire work.

Having discussed and interpreted all of the previous findings, I then integrate it all into a novel model that explains the observed effects of the P640T mutation. Here, I reframe and reapply the results in order to support my model, which I use to explicitly account for the findings of each experiment. In order to come up with this model, I did a lot of back-and-forth and bouncing of ideas with my lab partner, suggesting different plausible models and then figuring out how they would account for all of the findings. Any model's inability to account for a result meant to me that it was not accurate or substantial enough to be the correct one, leading me to ignore it altogether in order to identify a better-fitting model. Ultimately, I crafted a

model based mainly on the structural analysis that provided an explanation for all of the previous findings. However, because I could not yet verify this model, I outlined future experiments that would test my model’s accuracy.

Overall, this paper was a very valuable introduction to scientific writing for me, as I was motivated to adopt a different format than my previous literary writing while still keeping in mind all of the important factors that make effective writing. Writing this paper helped me realize that there can be a benefit to withholding analysis until all relevant data and information have been discussed, allowing the writer to take it all into account for an all-inclusive analysis, and I am honored to share my paper.

Professor Commentary

Aleksandra Snyder

Writing in the biological sciences requires a specialized skill set. Information transmitted to the reader is localized to individual sections of a scientific manuscript, each section, an abstract, an introduction, a materials and methods section, a results section and a discussion, serving a different purpose, yet each functioning with all of the other sections in a cohesive whole.

The introduction of a manuscript provides the context of the overall work and functions to capture the interest of readers who are bot familiar and unfamiliar with the topic discussed. In the introduction, the findings which are presented in the later results section of the paper, are related to previous findings in the field to “add to the story” of what has previously been found. The introduction does not serve as a full review of the overall topic being discussed but rather provides just enough information to understand the subject.

Ramie’s introduction to his manuscript examining the effect of a specific mutation in Msh2, a protein responsible for DNA mismatch repair, accomplishes this task well. He begins from a broad perspective to provide the relevance and excite the interest of the reader. He transitions from one paragraph to the next to specifically hone in on topic being studied while providing the context through the finding from previous studies. He ends with an elaboration of the objectives and findings of the research project presented.

In contrast to the introduction, the Discussion serves to interpret the results in the body of the manuscript, place the results in a broader scientific context and to propose new directions, hypotheses or models. Discussions typically have the opposite organization from the introduction, beginning with the specific findings and ending with broader implications and future directions. Previous published literature on the topic is brought into the discussion both in placing the results in the field as well as proposing future directions that have a sound basis in published work.

Again, Ramie's discussion of his data fits within the criteria of a discussion well. He does a nice job explaining what the data “means” beyond the most straightforward conclusions. He provides the most plausible explanations and introduces cogent arguments as to why he favors the interpretation put forth over another. He places his data in the context of the current literature on Msh2 mismatch repair and paints a complete picture of how the results presented add to the overall field. Lastly, he relates his findings from a yeast model of cancer to the human colorectal cancer perspective. Overall, Ramie's work represents a good model paper for writing in the biological sciences.